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Cytotoxic Effects of Acidic and Sulphur Containing Amino Acids on the Infant Mouse Central Nervous System

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Summary. The brains and retinas of infant mice were examined following subcutaneous administration of monosodium glutamate (MSG) and structurally related compounds in an attempt to clarify the molecular specificity of MSG-induced neuropathology. Based on the effects on the infant retina and hypothalamus all compounds could be placed into one of four groups: 1. Those equipotent with L-MSG in necrosing neurons. 2. Those substantially more potent than L-MSG in necrosing neurons. 3. Those which affect non-neuronal components (glial, ependymal, Muller cells) without appreciable effects on neurons. 4. Those with no cytotoxic effects. Except for L-cysteine, all neurotoxic compounds were acidic amino acids known to excite neurones, the most potent neurotoxic compounds being those which are powerful neuroexcitants (N-methyl DL-aspartic and DL-homocysteic acids). The exception posed by L-cysteine may be more apparent than real in that the *in vivo* conversion of the SH terminal to a more acidic group (SO_2H or SO_3H) could account for its neurotoxicity. The close correspondence in molecular specificities associated with neurotoxic and neuroexcitatory properties of simple amino acids suggests the two phenomena may be governed by similar mechanisms of action.

Key words: Cytotoxic Amino Acids — Infant CNS — Glutamate

Introduction

It has been demonstrated that subcutaneous administration to infant mice of monosodium L-glutamate (MSG) results in acute degeneration of cells in the inner layers of the retina (Lucas and Newhouse, 1957; Potts *et al.*, 1960; Cohen, 1967; Olney, 1969a) or the arcuate nucleus of the hypothalamus (Olney, 1969a, 1971a; Arees and Mayer, 1970; Burde *et al.*, 1971; Coulston, 1970). Originally, Lucas and Newhouse (1957) observed necrosis of retinal neurons following the administration of either L-glutamate or L-aspartate, but the aspartate effect was interpreted as being secondary to the *in vivo* conversion of aspartate to glutamate. Olney and Ho (1970) reported necrosis of neurons in the infant mouse retina and hypothalamus following oral intake of L-glutamate, L-aspartate or L-cysteine, but found no

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retinal or hypothalamic cytopathology following high oral doses of various other amino acids (glycine, L-serine, L-α-alanine, L-leucine, DL-methionine, L-phenylalanine, L-proline, L-lysine, L-arginine) or non-amino acid salts (sodium glutarate, sodium chloride). Identical results have been obtained by administering the same series of compounds subcutaneously (Olney, J.W. and Ho, O.L. unpublished).

The present study was undertaken to determine what effects subcutaneously administered compounds closely related structurally or metabolically to glutamate, aspartate or cysteine, might have on the infant retina and hypothalamus. D.R. Curtis and others (1960, 1963, 1965, 1969) have observed that a select group of simpe amino acids, including glutamate, aspartate and acidic congeners of cysteine and homocysteine, excite neurons when administered electrophoretically into the mammalian central nervous system. We have studied a number of these neuroexcitatory amino acids to provide a basis for evaluating the possibility that the molecular specificities underlying neuroexcitatory and neurotoxic properties of simple amino acids may be similar.

Material and Methods

Approximately 250 Webster Swiss albino mice (National Laboratory Animals, Missouri), 10 days old, were each given a single subcutaneous dose of one of 24 test compounds (Table 1). Because L-glutamic acid had previously been found predictably effective in producing conspicuous lesions with negligible mortality at a dose of 12 mmoles/kg, this dose was used initially with each compound tested. If a compound induced no cytopathic effects at 12 mmoles/kg it was also tested at 24 mmoles/kg. Several compounds were rapidly lethal to the infant mouse at 12 mmoles/kg, so that a pilot project was required with each such compound to empirically establish a sublethal dose range within which the neurotoxic potential of the compound could be tested. Each compound was tested on at least 4 animals after an appropriate dose range was established. All solutions were freshly prepared in distilled deionized water just prior to use, and were adjusted to pH 7.0 ± 0.2 with NaOH. Concentrations were varied so that the desired dose could be administered in a volume of approximately 0.1 ml. All animals were anesthetized with chloral hydrate and sacrificed by perfusion fixation 5 hours after treatment (or at serial intervals, including 5 hours, for a few compounds), and both the brains and retinas were processed for alternative examination by either light or electron microscopy according to a regimen described in detail elsewhere (Olney and Ho, 1970; Olney, 1971a). A method described previously (Olney and Ho, 1970) for roughly quantifying the pathological reaction in the infant hypothalamus was employed as an aid in comparing the neurotoxic potency of test compounds.

All compounds were obtained from Sigma Chemical Company, St. Louis, Missouri, except DL-homocysteic acid from Nutritional Biochemical Corporation, Cleveland, Ohio, and 2 amino 3 phosphonopropionic acid and 2 amino 4 phosphonobutyric acid from Calbiochem, Los Angeles, California. All animals used in these experiments were handled and cared for according to methods approved by the Council of the American Physiological Society.

Results

In terms of observed effect on the infant retina and hypothalamus, test compounds fell into one of four groups (Table 1A—D). It was relatively easy to group the compounds because for any given substance either a certain type of lesion consistently appeared in each animal appropriately dosed, or a complete absence of cytopathology was consistently evident. Minor exceptions were encountered with glutamine and asparagine which were ineffective at 12 mmoles/kg, but were weakly effective at 24 mmoles/kg. The cytotoxic potential of these compounds was clearly so weak that they were classified with the ineffective group (Table 1D).

Table 1. *Structure, cytospecificity and relative potency of compounds.* Severity of retinal and hypothalamic lesions following a 12 mm/kg dose of L-glutamic acid or DL-alpha aminoadipic acid were assigned a value of 1 for ease of expressing comparative potencies within each cytospecificity category. Minor differences in potency designated by (+) or (—)

Compound	Structure	Specificity	Pot.
A. L-glutamic acid	$HOOC-(CH_2)_2-CH(NH_2)-COOH$	N*	1
D-glutamic acid	$HOOC-(CH_2)_2-CH(NH_2)-COOH$	N	1 (+)
L-aspartic acid	$HOOC-CH_2-CH(NH_2)-COOH$	N	1
D-aspartic acid	$HOOC-CH_2-CH(NH_2)-COOH$	N	1 (+)
L-cysteine sulfinic acid	$HO_2S-CH_2-CH(NH_2)-COOH$	N	1 (+)
L-cysteic acid	$HO_3S-CH_2-CH(NH_2)-COOH$	N	1 (+)
L-cysteine	$HS-CH_2-CH(NH_2)-COOH$	N	1 (—)
B. N-methyl DL-glutamic acid	$HOOC-(CH_2)_2-CH(NHCH_3)-COOH$	N	5
N-methyl DL-aspartic acid	$HOOC-CH_2-CH(NHCH_3)-COOH$	N	100
DL-homocysteic acid	$HO_3S-(CH_2)_2-CH(NH_2)-COOH$	N	20
C. DL-alpha aminoadipic acid	$HOOC-(CH_2)_3-CH(NH_2)-COOH$	GEM**	1
alpha methyl DL-glutamic acid	$HOOC-(CH_2)_2-C(CH_3)(NH_2)-COOH$	GEM	1
alpha methyl DL-aspartic acid	$HOOC-CH_2-C(CH_3)(NH_2)-COOH$	GEM	1 (+)
2 amino 3 phosphonopropionic acid	$H_2O_3P-CH_2-CH(NH_2)-COOH$	GEM	1 (—)
2 amino 4 phosphonobutyric acid	$H_2O_3P-(CH_2)_2-CH(NH_2)-COOH$	GEM	1 (—)
D. alpha ketoglutaric acid	$HOOC-(CH_2)_2-CHO\ COOH$	–	0
L-glutamine	$H_2NOC-(CH_2)_2-CH(NH_2)-COOH$	–	0 (+)
gamma amino-n-butyric acid (GABA)	$HOOC-(CH_2)_3-NH_2$	–	0
N-acetyl L-glutamic acid	$HOOC-(CH_2)_2-CH(NHCOCH_3)-COOH$	–	0
DL-alpha aminopimelic acid	$HOOC-(CH_2)_4-CH(NH_2)-COOH$	–	0
L-asparagine	$H_2NOC-CH_2-CH(NH_2)-COOH$	–	0 (+)
N-acetyl L-aspartic acid	$HOOC-CH_2-CH(NHCOCH_3)-COOH$	–	0
N-acetyl L-cysteine	$HS-CH_2-CH(NHCOCH_3)-COOH$	–	0
3 mercaptopropionic acid	$HS-(CH_2)_2-COOH$	–	0

* N = Neuronal, ** GEM = Glial, Ependymal and Muller cell.

The first group (Table 1A) included L-glutamic acid (MSG) and other compounds judged approximately equipotent to it in causing acute neuronal degeneration. Lesions induced in either the retina (Fig. 4c) or hypothalamus (Figs. 1a—e) by these compounds were indistinguishable from those induced by MSG. This neuron-necrotizing type of reaction, as described in detail elsewhere (Olney, 1969a, 1971a; Olney and Sharpe, 1969), and further corroborated here, was characterized by massive swelling and organelle degeneration in postsynaptic elements, both dendritic and somatic (Figs. 2a—c). These changes occurred early, the first signs becoming evident within an hour of treatment. By approximately 3 hours, nuclear chromatin changes were evident; these evolved into frank pyknosis of the neuronal nucleus within an additional hour or two. Axon terminals or bundles of axons present in the arcuate region, but originating from cell bodies in other regions of the brain, retained a normal appearance while arcuate neurons in their midst were necrosed, phagocytized and evacuated from the region.

Despite their ability to necrose select neuronal populations in the infant central nervous system, each of the compounds in Table 1A was well tolerated at 12 mmoles/kg except L-cysteine which rendered treated animals practically moribund by the time of sacrifice. The excessive general toxicity of L-cysteine is not explained by its effect on hypothalamic neurons in that it tended to produce a slightly smaller hypothalamic lesion (Fig. 1c) than better tolerated compounds in the same group.

The second group (Table 1B) comprised those compounds found more powerful than L-glutamic acid as neuron-necrosing agents. The potency of these compounds, particularly N-methyl DL-aspartic acid, was reflected strikingly in the behavior of treated animals. Infant mice responded within seconds after a 12 mmoles/kg dose, exhibiting extreme irritability and wild running behavior which culminated in a sustained tonic seizure state and death within 1—2 min. Every dose of N-methyl DL-aspartic acid above 0.12 mmoles/kg was lethal, the time between treatment and death varying from 1—30 min depending on dose. Doses in the range of 0.06—0.10 mmoles/kg were usually non-lethal, but induced neuronal degeneration in the arcuate nucleus of the hypothalamus (Fig. 3) and in the inner layers of the retina. Both N-methyl DL-glutamic and DL-homocysteic acids were also rapidly lethal at 12 mmoles/kg. Scaling the dose of the former down to a range of 1.2—2.4 mmoles/kg, and the latter to a range of 0.3—0.6 mmoles/kg, resulted in acute neuronal degeneration in both the inner retina and arcuate nucleus of the hypothalamus without killing the animals. Based on doses required to produce lesions

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Fig. 1. *Hypothalamic effects of neurotoxic (a—f) and non-toxic (g—i) compounds compared by light microscopy 5 hours following treatment.* Each compound given at 12 mmoles/kg except DL-homocysteic acid at 0.3 mmoles/kg. a. L-glutamic acid. b. L-aspartic acid. c. L-cysteine. d. L-cysteine sulfinic acid. e. L-cysteic acid. f. DL-homocysteic acid. g. L-glutamine. h. N-acetyl L-cysteine. i. Alpha ketoglutaric acid. An acute lesion sweeping across the arcuate region but not extending into other hypothalamic regions is clearly evident in each section except those on the right (g—i) which appear normal (all × 80)

Fig. 2. *Ultrastructural features of hypothalamic lesion induced by L-glutamic acid (12 mmoles/kg).* a. Survey view of arcuate region 5 hours following treatment. Ependymal lining of third ventricle (III) and glial constituents about origin of median eminence (ME) appear normal but many cells of the arcuate nucleus are degenerating (× 500). b. Necrotic cell from rectangle in Fig. 2a. Signs of cellular degeneration include marked alterations in chromatin pattern of nucleus (arrow), vacuolization of endoplasmic reticular system (er), sphericalization of mitochondria (m) and diffusely distributed particulate debris. Identification of this cell as a neuron is based on the presence of an axosomatic synapse shown at higher magnification in Fig. 2c (× 7,000). c. Axosomatic synapse from neuron (Figs. 2a and b). Note displacement of presynaptic vesicles and membranes relative to post synaptic densification area (arrow), an early indication that the axon terminal (A) is separating from its contact with the cell surface (× 56,000)

Fig. 3. *Ultrastructural features of hypothalamic lesion induced by N-methyl DL-aspartic acid (0.06 mmoles/kg).* a. Survey view of arcuate region 5 hours following treatment. Normal ependymal and glial elements next to a field of degenerating arcuate neurons reveals the basic similarity of this lesion to that depicted in Figs. 2a—c following L-glutamic acid, despite the 100 fold difference in dose. Dark lines are artefact due to wrinkling of ultrathin section (× 600). b. Degenerating neuron from rectangle in survey scene (Fig. 3a). The same features of cellular degeneration are present in the nucleus (arrow), endoplasmic reticulum (er) and mitochondria (m) as were shown in Fig. 2b. Identification of cell as a neuron is based on synapses in boxed region which is magnified in Fig. 3c (× 4,800). c. Synapses terminating on cell surface of neuron shown in Figs. 3a and b. Note axon terminal at top is withdrawing from contact on cell surface as indicated by displaced position of post synaptic densification (× 44,000)

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Fig. 1

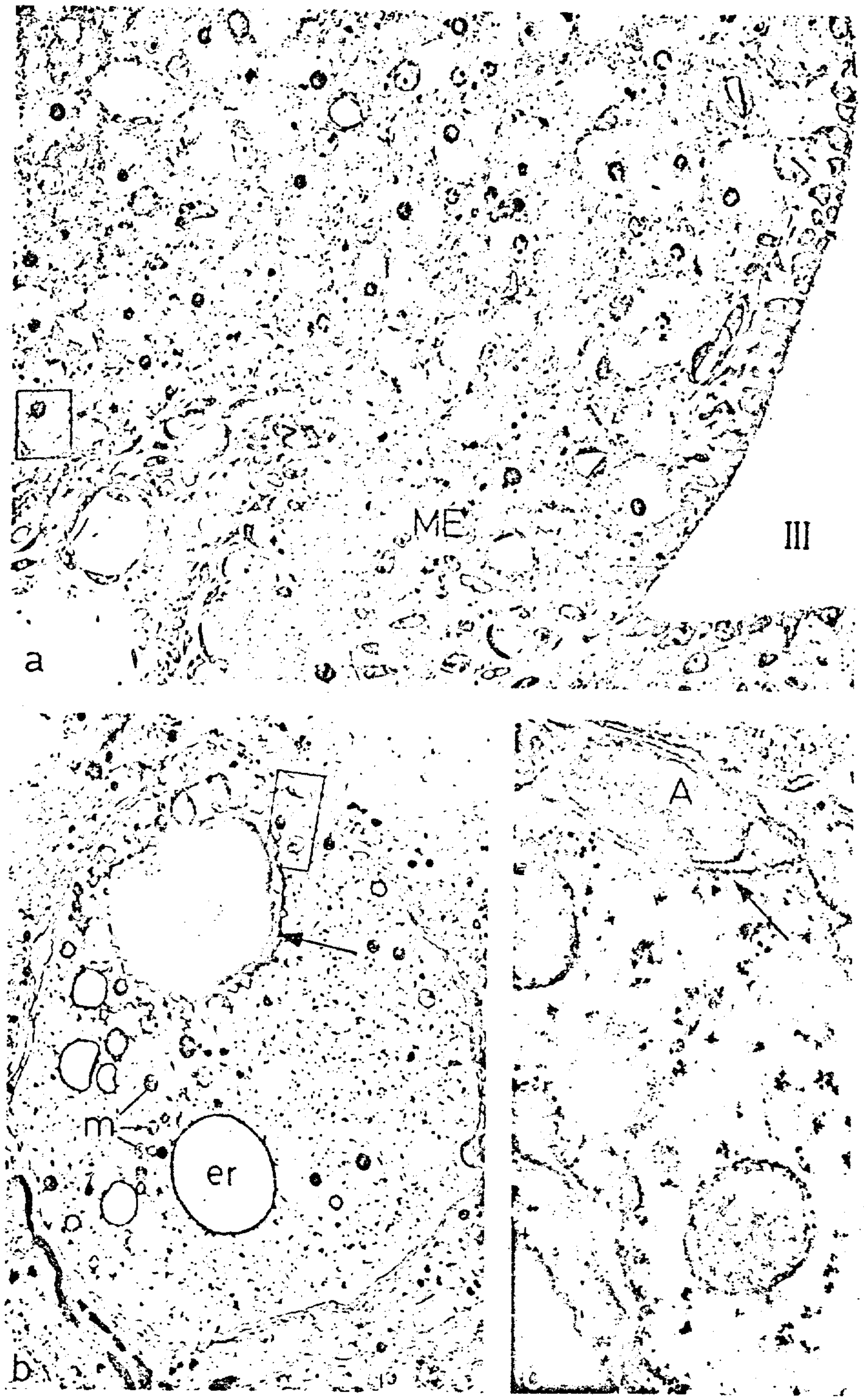

Fig. 2

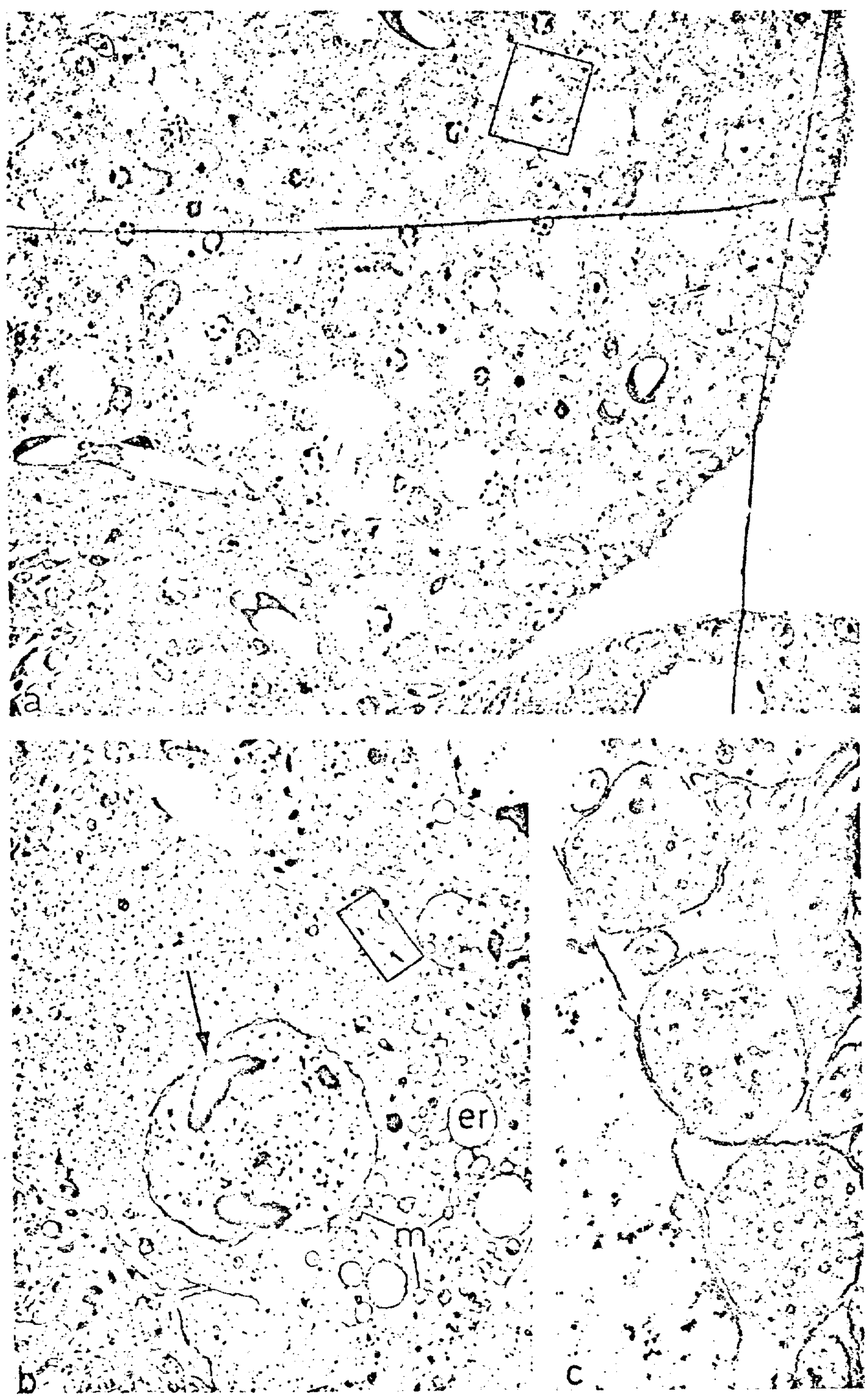

Fig. 3

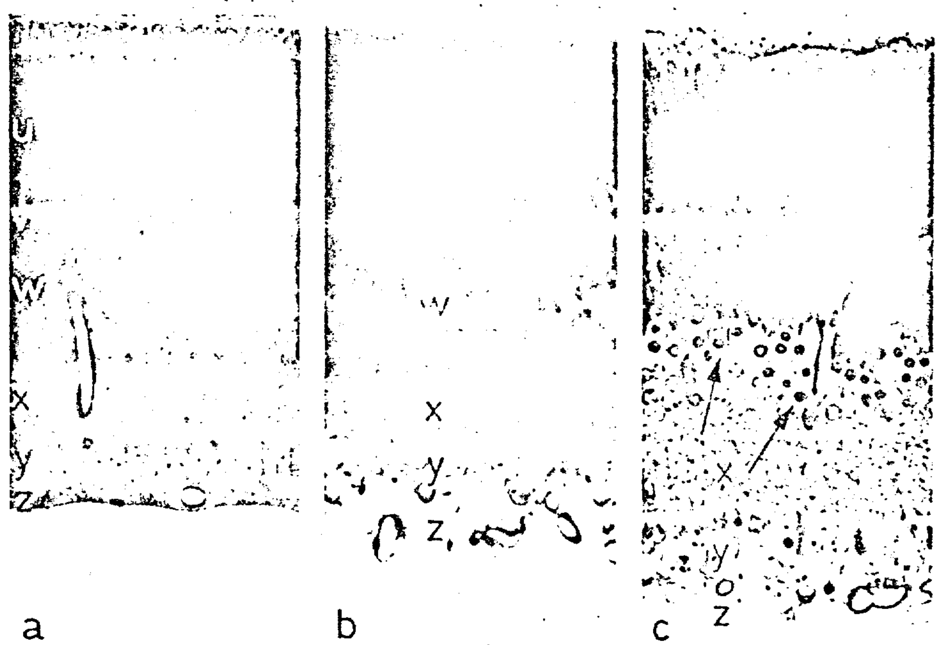

Fig. 4. *Comparison of retinas 5 hours after a non-toxic compound (a), a gliotoxic compound (b) and neurotoxic compound (c).* a. N-acetyl L-glutamic acid (12 mmoles/kg): the appearance of the infant retina is no different from that of an untreated animal (see illustration in Olney, 1969a). Retinal layers as follows: U = outer nuclear layer; V = outer plexiform layer; W = inner nuclear layer; X = inner plexiform layer; Y = ganglion cell layer; Z = Muller foot processes. b. Alpha aminoadipic acid (12 mmoles/kg): extreme edema of non-neuronal Muller cells is the only pathological manifestation. Swelling of nuclei and perikarya of Muller cells is evidenced by the light band running through middle of inner nuclear layer (W). Swollen foot processes (Z) of Muller cells cause widening of retina but note that row of ganglion cells (Y) surrounded by the Muller processes appear unaffected. c. L-cysteine (12 mmoles/kg); reaction pattern is identical to that following L-glutamic acid (illustrated in Olney, 1969a). Bipolar and amacrine neurons (arrows) in lower 1/3 of the inner nuclear layer, the ganglion cell layer (Y) and the neural processes comprising the inner plexiform layer (X) are degenerating. The extra thickness of this retina is due to edema of these inner retinal layers (all × 220)

of comparable size, N-methyl DL-aspartic acid was 100 times, DL-homocysteic acid 20 times and N-methyl DL-glutamic acid 5 times more potent than L-glutamic acid. By either light microscopy (Fig. 1f) or electron microscopy (Fig. 3) the cytopathological reaction pattern induced by these compounds appeared to be the same as that induced by L-glutamic acid and compounds of Table 1A.

The third group of compounds (Table 1C), at a dose of 12 mmoles/kg, induced a pathological reaction identical in regional distribution but differing in cytological specificity from that following treatment with L-glutamic acid (MSG) or other compounds in Tables 1A and B. The compound studied most extensively in this group, DL-alpha aminoadipic acid, induced changes in only one cell type of the retina, the non-neuronal Muller cell (Fig. 4b). Ganglion, amacrine and bipolar cells, which comprise the inner retinal neural constituents necrosed by MSG (Olney, 1969a) were not affected. Changes in the hypothalamus were restricted

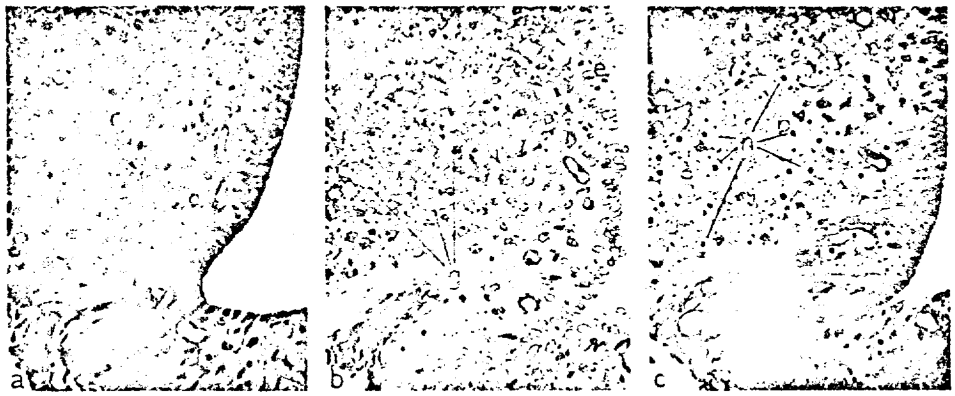

Fig. 5. *Light micrographic comparison of hypothalami 5 hours after a non-toxic compound (a) a gliotoxic compound (b) and neurotoxic compound (c).* a. N-acetyl L-glutamic acid (12 mmoles/kg); the arcuate region (ARC) of the infant hypothalamus appears essentially like that of an untreated animal (see illustrations in Olney, 1971 a). b. DL-alpha aminoadipic acid (12 mmoles/kg); marked edematous changes in glial (g) and ependymal (e) compartments distinguish this reaction pattern from the normal appearing hypothalamus (Fig. 5a) or from the pattern of rapid neuronal necrosis depicted in Fig. 5c. The fenestrated appearance of the arcuate region is caused by dilatation of glial processes surrounding the normal appearing neurons. c. L-glutamic acid (12 mmoles/kg); the numerous arcuate neurons (n) with dilated empty perikarya and shrunken dark nuclei beside an essentially normal ependymal wall give this reaction pattern a distinctive appearance which can be differentiated, even by light microscopy, from the glioependymal pattern in Fig. 5b (all × 300)

to the arcuate region, but appeared by light microscopy to be occurring around rather than within neurons (Fig. 5b). This reaction pattern was confirmed by electron microscopic examination at several intervals after treatment during which glial and ependymal cells manifested marked edema, progressing in some cells to organelle degeneration and cellular necrosis, while neurons retained a normal appearance (Figs. 6b, 7 and 8). Synaptic involvement is the most useful criterion available for distinguishing neuronal from non-neuronal cellular elements in acutely degenerating tissues of the central nervous system. This is particularly true in the arcuate nucleus, where neurons all fall within a size range only slightly larger than glia. Although we found many edematous and degenerate appearing structures in the arcuate region of animals treated with alpha aminoadipic acid, none was involved in a synapse.

The phosphonic acid analogues and the alpha methylated (methyl group on the alpha C rather than N atom) derivatives of DL-glutamic and aspartic acids (Table 1C) also tended to induce gliotoxic but neuron-sparing reactions, although the separation of effect between neuronal and non-neuronal compartments was most complete following alpha aminoadipic acid (other compounds in the group preferentially affected non-neuronal components but also affected neuronal components slightly, especially the alpha methylated compounds.

The final group (Table 1D) comprised those compounds which were judged to have essentially no cytotoxic action on the infant hypothalamus (Figs. 1g—i, 5a and 6a) or retina (Fig. 4a). Each compound in this group was tested initially at 12 mmoles/kg, and when observed to be ineffective at this dose was tested also at

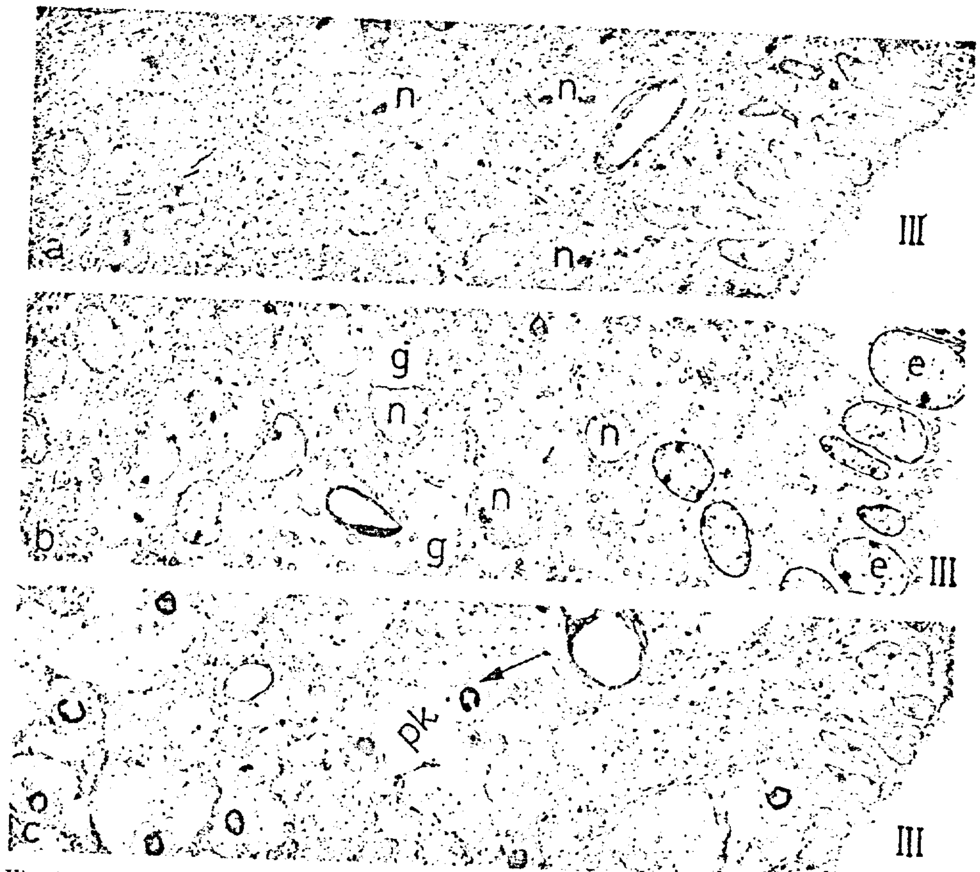

Fig. 6. *Survey ultrastructural views of the arcuate nucleus 5 hours after non-toxic (a), gliotoxic (b) and neurotoxic (c) compounds.* a. N-acetyl L-glutamic acid (12 mmoles/kg); neurons (n), neuropil areas and the ependymal lining of the 3rd ventricle (III) all appear normal. b. Alpha aminoadipic acid (12 mmoles/kg); cell bodies and nuclei of neurons (n) appear normal amidst massively swollen glial processes (g). Note the extreme tumefaction of ependymal nuclei (e). c. L-glutamic acid; the pyknotic nuclei (arrows) of neurons are surrounded by massively edematous neuronal perikarya (pk) but the ependymal lining of the 3rd ventricle (III) and portions of the neuropil which are maximally affected in Fig. 6b are not involved (all × 900)

24 mmoles/kg. An exception was 3 mercaptopropionic acid which was consistently lethal at 12 mmoles/kg and, therefore, was not studied at 24 mmoles/kg. This non-amino acid homologue of cysteine induces seizures in adult rodents (Sprince *et al.*, 1969). In the dose range of 0.06—0.6 mmoles/kg severe repeated opisthotonic seizures were induced in infant animals, and this was followed by prolonged hyper-excitability, but at 6 and 12 mmoles/kg the animals became prostrate and died over a 1—4 hour period without having seizures. Despite the convulsant activity of this compound at low doses, and severe general toxicity at higher doses, it did not induce necrosis of retinal or hypothalamic neurons. Glutamine and asparagine, the amide forms of glutamic and aspartic acids respectively, were ineffective at 12 mmoles/kg but consistently necrosed a small number of neurons at 24 mmoles/

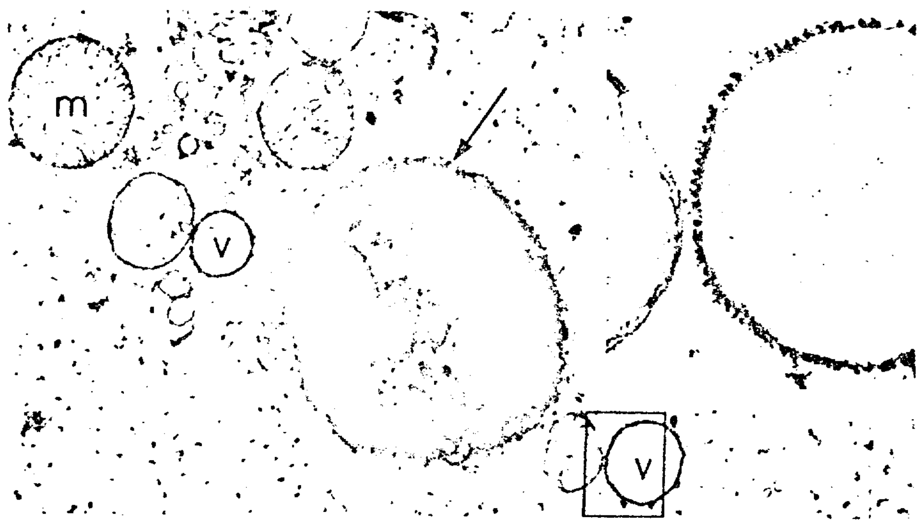

Fig. 7. *Glial degeneration 10 hours after DL-alpha aminoadipic acid (12 mmoles/kg).* The neuron (n) appears unaffected with a normal nuclear chromatin pattern and mitochondria (m) of normal size, density and shape. Pathological changes in the glial cell (g) include peripheralization of nuclear chromatin, hydropic cytoplasm and massive spherical mitochondria (m) measuring 10—15 times the diameter of normal mitochondria (× 9,000)

Fig. 8. *Glial degeneration 12 hours after DL-alpha aminoadipic acid (12 mmoles/kg).* a. Mitochondria (m) remain massively dilated, rough surfaced vacuoles (v) have formed (see inset) and the nucleus (arrow) has reached an advanced stage of pyknosis (× 12,000). Inset from boxed region illustrates granules adhering to the outer surface of a membrane-bound vacuole. This type of vacuole, probably originating from endoplasmic reticulum, is also seen in degenerating neurons following treatment with L-glutamic acid (× 46,000)

kg. It seems reasonable to explain this effect in terms of the *in vivo* conversion of these compounds to glutamic and aspartic acids. Gamma amino butyric acid (GABA) at both 12 and 24 mmoles/kg had essentially a sedating effect, but produced no changes in retinal or hypothalamic neurons.

Discussion

Several authors have reported data on the subject of MSG-induced brain damage which are inconsistent with findings from this laboratory. Adamo and Ratner (1970) postulated species variation to account for their failure to observe brain damage in adult rats treated with MSG in infancy. However, Arees and Mayer (1970), Burde *et al.* (1971) and Olney (1969 b) have all found the rat, as well as the mouse, susceptible to MSG-induced brain damage. Multiple endocrine disturbances (Redding and Shally, 1970: Redding *et al.*, 1971) and obesity (Knittle and Ginsberg-Feller, 1970) have also been reported in MSG-treated rats. Methodological variables which might explain the failure of Adamo and Ratner to detect an MSG effect in the rat have been discussed elsewhere (Olney, 1971 b).

Oser and colleagues (1971), employing 3 species of experimental animal (mice, rats, dogs), recently explored the toxic potential of MSG from a food safety standpoint. The failure to detect brain damage in any of the 3 species following MSG treatment probably relates to certain features of the research design. For example, the treatment schedule was limited to a single minimally effective dose of MSG, a feeding tube was not used to assure that orally treated animals actually received the full dose described, brains were not examined in early post treatment intervals when cytopathological changes are known to be most conspicious and readily interpreted, and relatively unrefined techniques were used for tissue preparation. When Burde *et al.* (1971) treated infant mice by feeding tube with the same oral dose of MSG employed by Oser and colleagues (1 mg/gm), but sacrificed animals within 5 hours after treatment and replicated the histolocial procedures described by Olney (1971 a) and Olney and Ho (1970), acute degeneration of neurons was detected in the arcuate nucleus of all MSG-treated infants and in none of the NaCl-treated controls.

Arees and Mayer recently were able to demonstrate, with several histopathology techniques, that neuronal degeneration occurs in infant mouse brain following subcutaneous treatment with MSG (Arees *et al.*, 1971; Arees, E.A. personal communication) and, therefore, have discarded their prior interpretation (Arees and Mayer, 1970) that MSG treatment affects "microglia" rather than neurons.

In electrophoretic studies with simple amino acids it has been established (Curtis and Watkins, 1960, 1963, 1965; Curtis and Crawford, 1969) that neuroexcitatory properties are primarily confined to select amino acids conforming to the general molecular structure

$$X\text{—}(CH_2)_n\text{—}CH(NH_2)\text{—}CO_2H,$$

where X may be CO_2H, SO_2H or SO_3H and $n = 1$ or 2. D and L enantiomorphs were usually of equal strength, and alkyl substitution within the carbon chain or on the N atom is usually associated with diminution or loss of excitatory activity. However, these generalizations are subject to the exceptions that N-methyl D-aspartic and D-homocysteic acids were exceedingly potent neuronal excitants, the former being the more potent of the two.

Our data indicate that the same general rules may apply for predicting neurotoxicity from the structural formula of a simple amino acid as apply for predicting neuroexcitatory activity. Further, it appears that the same major exceptions are applicable in that two structural modifications known to markedly increase rather than decrease neuroexcitatory potency (N-methyl DL-aspartic and DL-homocysteic acids) also resulted in marked increases in neurotoxicity. It seems likely that the extreme neurotoxicity of the racemic preparations of N-methyl aspartic and homocysteic acids was due to the presence in each case of the D isomer. However, this point cannot be considered well established until L and D forms of each compound are separately evaluated for neurotoxicity. Other modifications known to reduce neuroexcitatory activity were associated in these experiments with neuron-sparing but gliotoxic effects, and an assortment of amino acids with no known neuroexcitatory properties were also found to have no cytotoxic effects.

Possible exceptions to the close correspondence in structure-activity relationships for neuroexcitatory and neurotoxic amino acids are N-methyl DL-glutamic acid and L-cysteine. N-methyl DL-glutamic acid is probably a very minor exception. It was about 5 times more effective than either D or L-glutamic acid in neurotoxic action by our evaluation, but was rated slightly less potent than D or L-glutamic acid as a neuroexcitant in electrophoretic studies in the cat (Curtis and Watkins, 1963). It has been shown, however, that N-methyl DL-glutamic acid is more potent than L-glutamic acid, and much less potent than N-methyl DL-aspartic acid, in producing excitation and convulsions when administered to young adult mice by intraventicular injection (Crawford, 1963). This correlates well with our findings regarding the relative neurotoxic potencies of these compounds in infant mice. Species, age or topographical variables might explain the minor differences between our findings and electrophoretic data for N-methyl DL-glutamic acid.

In our experiments L-cysteine was tolerated poorly and induce neurodegenerative changes in infant retina and hypothalamus. Yet, the terminal SH group of cysteine is not acidic like the CO_2H, SO_2H or SO_3H terminals associated with neuroexcitatory activity, nor has L-cysteine been demonstrated to have neuroexcitatory activity in electrophoretic experiments (Curtis, D. R. personal communication). Furthermore, we have observed that a disseminated pattern of neuronal degeneration appears over a 24 hour period following the subcutaneous administration of L-cysteine which involves areas of brain (cerebral cortex, dorsal hippocampus, thalamus, midbrain) not affected by subcutaneously administered acidic amino acids (Olney, J.W., unpublished observations). A tentative way of reconciling these observations would be to assume that L-cysteine gains entry to many more portions of the infant brain than do the acidic amino acids, but then becomes toxic by conversion locally within the brain to one of its acidic analogues (cysteine sulfinic or cysteic acids).

Since the molecular specificities associated with neuroexcitatory and neurotoxic properties of amino acids are very similar, if not identical, there is a strong possibility that the two phenomena are mediated by a common mechanism of action. It is assumed from electrophoretic experiments that the acidic amino acids interact with external receptors of neuronal membranes, but it remains to be established whether such an interaction is confined to the synaptic region (Curtis, 1969). We observed that postsynaptic (dendritic and somal), but not presynaptic (axonal), components were among the earliest neural elements to react. This is consistent with the postulate that glutamate or other acidic amino acids (Curtis, 1969) function physiologically as neurotransmitters, since it would be expected that the postsynaptic region would be particularly sensitive to increased extracellular concentrations of such compounds. Sustained high concentrations infiltrating the synaptic cleft region could lead to persistent depolarization, altered ionic permeability of neural membranes and, conceivably, to neuronal necrosis. Alternatively, however, our observations are also consistent with a direct effect of acidic amino acids on extrasynaptic neural membranes, since this could produce a similar sequence of events and, in either case, whole dendrites and cell bodies as well as synaptic regions might manifest changes as early concomitants of neuronal degeneration. The fact that certain of the acidic amino acids tested were gliotoxic but not neurotoxic suggests that at least some acidic amino acids can produce cytotoxic effects on the basis of a mechanism not mediated through synaptically specialized membranes. Of course, this does not preclude a selective effect on neurosynaptic membranes by one species of acidic amino acid, and an effect specific for synaptically unspecialized glial membranes by another. In fact, since gliotoxic effects were particularly well defined but surrounding neurons were completely unaffected following treatment with alpha aminoadipic acid, and this compound is a natural metabolite of brain (Takao and Kanazawa, 1966), a role is suggested for alpha aminoadipic acid in the functioning of glial membranes which parallels the role that glutamate or other neuroexcitatory compounds might have in relation to neural membranes.

Since subcutaneous administration of neuroexcitatory amino acids induced pathological changes only in cells of the retina and arcuate nucleus of the hypothalamus, the question arises whether the mechanism of toxicity is selective for these neuronal populations or is one to which neurons in general might be vulnerable. We think the latter is the case, because we have found that doses of MSG substantially higher than 12 mmole/kg induce lesions in certain extrahypothalamic regions of the brain (Olney, 1969b), and it is possible with high MSG doses (24 mmole/kg) to produce a brain lesion in the 1 day old mouse which spreads beyond the arcuate nucleus to involve indiscriminately neurons lying within a wide arc about the mediobasilar portions of the brain (Olney, J.W. unpublished). Further, we have found (Perez, V. and Olney, J.W., unpublished observations) that the glutamic acid concentration of the arcuate nucleus of the mouse hypothalamus increases to 4 times normal levels following subcutaneous administration of MSG, while the adjacent ventromedial nucleus and other brain regions show no substantial increase in glutamic acid concentration. This suggests that arcuate neurons are selectively necrosed by the neuroexcitatory amino acids simply because they are a group of brain cells peculiarly exposed to the subcutaneous route of administration. Also relevant is the observation that L-cysteine, in addition to its selective early effect on the hypothalamus and retina, is capable of producing more widely disseminated neurodegenerative effects, possibly on a basis of delayed conversion to more acidic analogues in various other portions of the brain.

Taken together these several lines of evidence suggest that many neuronal populations outside the retina and hypothalamus are susceptible to the toxic mechanism of the acidic amino acids. Further, the neurotoxicity of acidic amino acids appears to cut across most species boundaries since each species tested for MSG-induced hypothalamic damage (mice, rats, rabbits, chicks, monkeys) has been susceptible (Olney, J.W., unpublished observations). It may be important, therefore, that several of these amino acids are natural metabolites of the central nervous system (glutamic, aspartic, cysteine sulfinic and cysteic acids). Since even a transient elevation in the brain concentration of one or more of these natural metabolites could conceivably trigger a neurodegenerative syndrome in the developing human brain, the potential role of acidic amino acids in the pathogenesis of aetiologically obscure syndromes of mental retardation or other forms of aberrant mental functioning warrants consideration.

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Brain Lesions, Obesity, and Other Disturbances in Mice Treated with Monosodium Glutamate

Abstract. *In newborn mice subcutaneous injections of monosodium glutamate induced acute neuronal necrosis in several regions of developing brain including the hypothalamus. As adults, treated animals showed stunted skeletal development, marked obesity, and female sterility. Pathological changes were also found in several organs associated with endocrine function. Studies of food consumption failed to demonstrate hyperphagia to explain the obesity. It is postulated that the obesity syndrome represents a multifaceted neuroendocrine disturbance arising from the disruption of developing neural centers concerned in the mediation of endocrine function.*

Parenterally administered monosodium glutamate (MSG) produces an acute degenerative lesion in the inner retina of normal neonatal mice (*1*). Although the acute lesion has been described both light and electron microscopically (*2*) and several biochemical parameters have been studied (*3*), the specific mechanisms underlying the effect of MSG on retinal neurons have not been definitively clarified. That MSG treatment might have a similar deleterious effect on neurons in other regions of the central nervous system (CNS) has apparently not been considered. A suspicion that hypothalamic damage might be associated with glutamate treatment was aroused by the observation that several months after neonatal mice were treated with glutamate, for purposes of inducing retinal pathology (*4*), they became quite obese. Data establishing that glutamate treatment does induce brain lesions are here presented, and a preliminary characterization is given of a syndrome resulting from glutamate treatment which features obesity as its most striking characteristic.

Ten litters of Swiss albino mice, 2 to 9 days old, were killed from 1 to 48 hours after a single subcutaneous injection of MSG (dosages varied from 0.5 to 4 mg/g), and brains were examined by light microscopy for acute pathology. Brain lesions characterized by intracellular edema and neuronal necrosis developed within a few hours of treatment at every dose tested, including 0.5 mg/g (Fig. 1a). Certain structures located in a paramedian plane and bordering on the roof and floor of the third ventricle were preferentially affected. At the base of the brain, preoptic and arcuate nuclei of the hypothalamus were selectively destroyed along with scattered neurons within the median eminence (nuclei tuberales). No acute changes were found in other hypothalamic areas or in the pituitary. Dorsally, the subcommissural and subfornical organs and neuronal groups contiguous with them were involved, including the medial habenular nuclei and neurons of the rostral hippocampus (dentate gyrus). Acute lesions were also found in brains of adult mice given high doses (5 to 7 mg/g) of MSG subcutaneously (Fig. 1b). Whether lower dosages than those tested might induce neuronal pathology in either the immature or mature CNS requires further systematic investigation. Brain lesions were also found in the C57BL/6 strain of mice and in albino rats after MSG treatment in the neonatal period.

To study the possibility of long-range effects accruing from glutamate treatment of the neonate I followed five litters of Swiss albino mice, consisting of 38 healthy animals, from birth to 9 months of age. Twenty animals received subcutaneous injections of MSG daily from 1 to 10 days after birth, according to a dose schedule described by Cohen (*4*); 18 controls received no treatment. All animals were weighed individually on a weekly

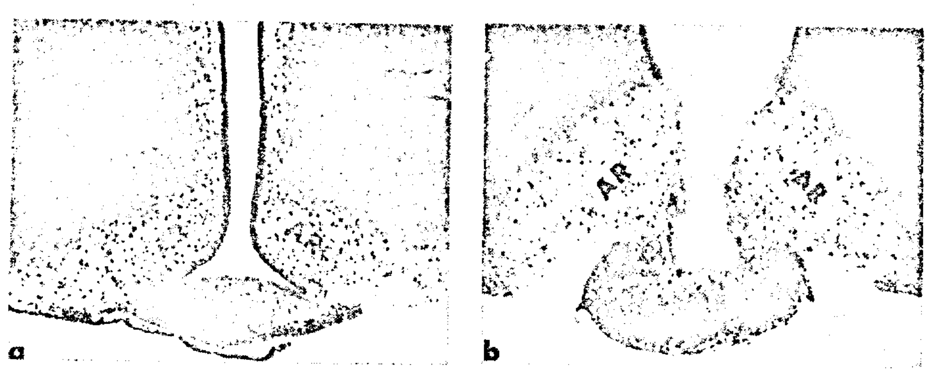

Fig. 1. (a) Section through hypothalamus of 5-day-old Swiss albino mouse showing lesion formation 3 hours after a subcutaneous dose of MSG (1 mg/g). Scattered neurons in the median eminence (*ME*) are necrotic with bloated cytoplasm and pyknotic nuclei. The majority of neurons in the arcuate nuclei (*AR*) are necrotic, but those of the ventromedial nuclei (*VM*) are unaffected (× 100). (b) Section through hypothalamus of adult C57BL/6 mouse 3 hours after a subcutaneous dose of MSG (6 mg/g). The arcuate nuclei (*AR*) are completely destroyed along with neuronal constituents in the median eminence (*ME*). Capillary lumina are empty and widely dilated because this animal was killed by perfusion of glutaraldehyde through the ascending aorta (× 115).

basis from the 1st to 5th months, and data on food consumption were gathered weekly on all males for the same period. Animals were given free access to Purina mouse breeder chow in containers designed to avoid food loss by spillage or soiling of food by urine and feces.

Treated animals appeared stunted at termination of treatment on the 10th day after birth and remained smaller than controls on the 30th day. Data on growth presented separately for females (Fig. 2a) and males (Fig. 2b) illustrate that the rate of weight gain for experimental animals was greater than for controls throughout the period from 30 to 150 days. Treated animals surpassed controls in weight at a mean age of approximately 45 days, and at 150 days the range of weights for treated animals showed no overlap with those of controls. Experimental females in this series gained more weight by comparison to controls of their own sex than did treated males.

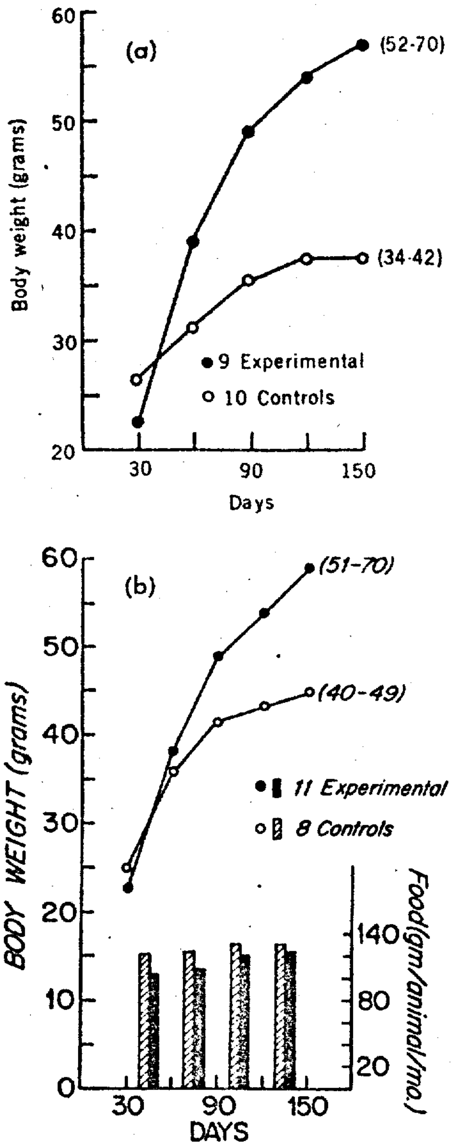

Fig. 2. (a) Composite growth records for experimental and control females from five litters of mice covering the 1st to 5th months of life. Weight ranges on the 150th day are in parentheses. (b) Composite growth records and data on food consumption for experimental and control males from five litters of mice covering the 1st to 5th months of life. Ranges of weight on the 150th day are in parentheses.

Data on food consumption compiled for all males of the five litters studied are included in Fig. 2b for direct comparison with their growth data for comparable periods. Contrary to expectation, treated animals consumed slightly less food than controls in every period for which data were collected. Food consumption was also measured on all animals, both male and female, from the same five litters for 4 hours of unrestricted eating after the 24 hours during which food was withheld. Mean per capita consumption over the 4 hours was 2.5 g for controls and 1.7 g for experimental animals.

Treated animals continued to gain weight on unrestricted diets beyond the age of 5 months (Fig. 3). Despite weight excesses, however, treated animals were approximately 10 percent shorter in mean body length than controls. These differences were reflected in measurements of the long bones and spines of animals x-rayed at 9 months. Treated animals were quite lethargic as adults, and they lacked the sleekness of body coat seen in controls (Fig. 3). The reproductive capacity of treated females was also affected in that they consistently failed to conceive in spite of adequate exposure, from 5 to 9 months of age, to both treated and normal males. Mating of treated males with untreated females resulted in pregnancies and normal offspring.

Autopsies performed at 9 months of age revealed massive accumulations of adipose tissue in experimental animals compared with small to moderate amounts in controls. The livers of treated animals showed fatty changes, and the ovaries contained approximately twice as many atretic follicles as were found in controls. The uteri of treated animals were easily distinguished from controls by their slender, attenuated appearance. The endometrium was thinner and contained only small secretion-poor glands. The testes of treated males were indistinguishable from controls. Findings suggestive of a mild adrenocortical hypertrophy remain under study at this time. The pars nervosa and pars intermedia of pituitary glands from experimental animals appeared normal but an overall reduction in mass and in the number of cells was evident in the pars distalis (adenohypophysis).

Fig. 3. A 9-month-old Swiss albino mouse (left) which was treated, as a newborn with MSG is shown beside the heaviest untreated male (right) from the same litter. The experimental animal weighed 84 g compared to 44 g for the control. In addition, the treated animal is shorter than the control, and his body coat is not as sleek as that of the control.

Three additional litters of Swiss albino mice (10 experimental and 11 control animals) were used to test the obesity-inducing potential of a single subcutaneous injection of MSG (4 mg/g) 2 days after birth. Treated animals in this series were on the average 16.9 g heavier than littermate controls at 9 months of age. However, this represented a more slowly developing and less severe syndrome than was created by daily treatments for the first 10 days of life.

These observations, linking MSG treatment of the neonatal mouse with a syndrome of manifestations, including skeletal stunting, marked adiposity, and sterility of the female, coupled with histopathological findings in several organs associated with endocrine function, suggest a complex endocrine disturbance. In view of the additional finding of lesions in regions of the brain thought to function as neuroendocrine regulatory centers (5), a unitary hypothesis might be constructed relating all or most of the findings to the neonatal disruption of neuronal development in these centers. Since acute degenerative changes were not found in neonatal pituitary glands, the small size of adult pituitary glands from treated animals suggests an interference in some extrapituitary influence (perhaps hypothalamic) on the development of this gland.

Obesity, the most striking clinical manifestation of MSG treatment, has been produced experimentally in mice treated with two other chemical compounds, gold thioglucose (GTG) (*6*) and bipiperidyl mustard (*7*). In each case, however, animals were treated in adulthood, lesions were reported in the ventromedial nucleus ("satiety center") of the hypothalamus, and treated animals were considered hyperphagic. In that hypothalamic lesions in MSG-treated animals routinely spared ventromedial nuclei and these animals were consistently hypophagic by comparison with littermate controls, a mechanism other than appetite disturbance must be considered. Whether a regulatory mechanism affecting fat metabolism in the mouse can be localized to the arcuate nucleus, or other brain areas selectively destroyed by MSG treatment, requires further study.

The assumption that MSG is an entirely innocuous substance for human consumption has been questioned recently in view of its role in the Chinese restaurant syndrome (*8*). The finding that neuronal necrosis can be induced in the immature mouse brain by 0.5 mg/g of MSG raises the more specific question whether there is any risk to the developing human nervous system by maternal use of MSG during pregnancy. The primate placenta maintains amino acids in consistently higher concentrations in the fetal circulation than are found in the maternal circulation, the ratio for glutamic acid being greater than 2:1 (*9*). In fact, when high doses of phenylalanine were given to a pregnant rhesus monkey, the ratio of mother to fetus for this amino acid remained unchanged so that exceedingly high fetal blood levels resulted (*9*). The possibility that brain lesions could occur in the developing primate embryo in response to increased glutamic acid concentrations in the maternal circulation, therefore, warrants investigation.

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Brain Lesions in an Infant Rhesus Monkey Treated with Monosodium Glutamate

Abstract. *In an infant rhesus monkey brain damage resulted from subcutaneously administered monosodium glutamate. Although a relatively high dose of monosodium glutamate was used, the infant was asymptomatic for a 3-hour observation period during which time hypothalamic neurons were undergoing a process of acute cell death. With the electron microscope it was observed that dendrites and cell bodies of neurons are the tissue components primarily affected in brain damage induced by monosodium glutamate.*

Susceptibility of the developing central nervous system to damage from subcutaneously administered monosodium glutamate (MSG) has been observed in every species of experimental animal tested thus far—mice (*1, 2*), rats (*2, 3*), and rabbits (*4*). In mice, which have been studied more extensively for MSG-induced brain damage than other species, the lowest effective dose for the baby animal (0.5 g/kg) was approximately one-tenth that for the adult (5 g/kg) (*2*). Additional studies are needed to clarify mechanisms underlying the MSG effect and to elucidate the basis of enhanced vulnerability on the part of the immature nervous system. In the meantime, the question arises whether glutamate could have an occult etiologic involvement in any of the unexplained brain damage syndromes occurring in the course of human ontogenesis and whether the widespread practice of feeding glutamate-enriched diets to human infants is a wise one (*5*). The feasibility of studying these questions in the primate is suggested by our evidence that the infant rhesus monkey (*Macaca mulata*) is susceptible to glutamate-induced brain damage.

Our report is based on only one test subject because we were unable to obtain additional baby monkeys at this time. However, the pattern of neuronal necrosis induced in the hypothalamus of experimental animals by MSG is highly selective for certain cell types and has a very distinctive appearance. Furthermore, as a frame of reference, we have extensive light and electron microscopic data pertaining to the evolution of this type of lesion in the retina (*6*) and the hypothalamus (*7*) of numerous rats and mice. The fact that the margins of the MSG lesion are sharply demarcated was helpful for evaluating fixation variables with the electron microscope because normal, well-fixed cells of every kind typical for a given region could be examined, just beyond the margin of the lesion, for comparison with degenerating cells within the damaged area.

We separated an infant rhesus monkey from its mother 8 hours after birth; the infant was an alert, healthy-appearing male with an active cry and appropriate spontaneous motor activity. However, it weighed only 260 g and measured 16.5 cm from crown to rump, so that, judged by size, it would probably be classified as a premature infant (8). Glutavene, a commercially available preparation of MSG in 25 percent aqueous solution, was injected subcutaneously in a volume of 2.8 ml, the total dose being 0.7 g or 2.7 g per kilogram of body weight. The treated infant was then held and cared for in a maternal manner (but not provided with food) for a 3-hour observation period; during this time there were no manifestations of a central nervous system disturbance. Three hours after treatment the infant was given 1 mg of Sernylan (Parke, Davis) intramuscularly, which provided excellent anesthesia characterized by a deep sleep with loss of responsiveness to painful stimuli but with retention of full rhythmical respirations. Thoracotomy was then performed so that a cannula could be clamped into the ascending aorta,

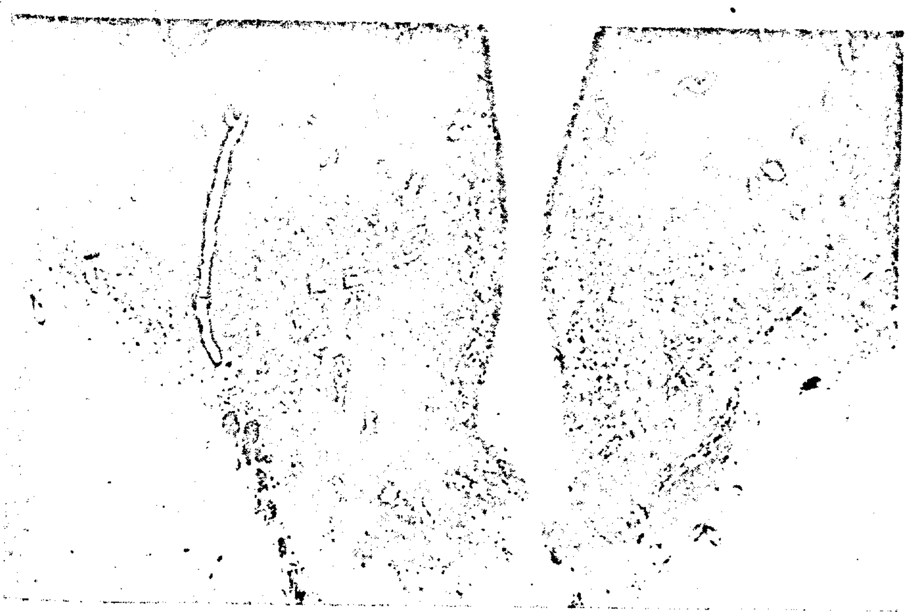

Fig. 1 (top). Cross section of the ventral hypothalamus cutting through the infundibular stalk. The lesion (*LES*) affects the periventricular-arcuate regions bilaterally, giving these areas a rarefied appearance. A "Swiss cheese effect" is created by the dilatation of dendritic processes. The larger holes and open spaces are dilated blood vessels resulting from perfusion fixation (× 50).

Fig. 2 (bottom left). An electron micrograph showing a massively dilated dendritic process (*d*) in synaptic contact with a normal-appearing axon terminal (*a*). The internal content of the dendrite consists primarily of diffusely distributed particulate debris. The axon is not swollen and contains numerous synaptic vessicles and normal-appearing mitochondria (× 10,300).

Fig. 3 (bottom right). An electron micrograph of two degenerating neurons (*a* and *b*) illustrating alteration of nuclear chromatin pattern (arrow, *a*) and disintegration of cytoplasmic components. The membrane system comprising the endoplasmic reticulum has degenerated beyond recognition and mitochondria have either ruptured or become completely spherical (× 6000).

and perfusion of the brain was begun within 30 seconds. The perfusate consisted of 3 percent glutaraldehyde in $0.1M$ cacodylate buffer and 0.02 percent $CaCl_2$. After 20 minutes of perfusion, the brain, pituitary gland, eyes, and optic nerves were removed and placed in jars containing the perfusion fluid. Areas of special interest were dissected out from these tissues which were then fixed further in osmium tetroxide for 2 hours, dehydrated in graded ethanols, and embedded in Araldite after an intermediate stage in toluene. Sections 1 μm thick were cut with glass knives (0.95 cm) and stained for light microscopy (*9*). Sites of lesion formation identified by light microscopy were examined with the electron microscope in ultrathin sections prepared from the same block.

A lesion affecting the periventricular-arcuate region of the hypothalamus and essentially identical in light microscopic appearance to the form of pathology seen in mouse brain after MSG treatment (*2, 7*) was readily apparent (Fig. 1). Electron microscopic examination established that the cellular constituents primarily affected were dendrites and cell bodies of neurons. Many synaptic complexes could be found in which the postsynaptic (dendritic) component was massively dilated (Fig. 2). These processes were either empty or contained degenerating organelles and diffusely distributed particulate debris. The presynaptic component (axonal) of such complexes was usually unaffected, as were axon bundles passing through the region of injury. Many neuronal cell bodies were swollen with intracellular edema and, in some, the cytoplasmic organelles appeared to have undergone a lytic process, while nuclei showed marked alterations in chromatin pattern (Fig. 3). A mild intracellular edema of the ependyma was evident, but this was not accompanied by degenerative changes in either nuclei or intracellular organelles, and no alterations were noted in the appearance of junctional complexes between ependymal cells. No structural alterations were detected in glial or vascular components to suggest involvement of these elements in the pathological process.

The lack of symptoms in this primate infant during the time when a small percentage of its brain cells were being destroyed is evidence of a subtle process of brain damage in the developmental period, which could easily go unrecognized were it to occur in the human infant under routine circumstances. However, a high dose of MSG was used to produce brain damage in this neonatal monkey, and it was administered by the subcutaneous rather than oral route. Thus, while we have demonstrated susceptibility of a primate species to the mechanism of the glutamate effect, it remains to be seen whether this mechanism can be triggered by any set of naturally occurring circumstances. Presumably, an elevated blood concentration of glutamic acid is an important prerequisite to lesion formation.

In attempting to evaluate the risk of glutamic acid blood concentrations rising high enough to produce brain damage in the human infant, it is important to recognize that the oral dose of MSG is but one among several potential determinants of glutamic acid concentrations in the blood. Other factors, such as circadian periodicity (*10*), viral infection (*11*), immaturity of enzyme systems, rapid absorption from an empty gastrointestinal tract, and individual variations in metabolic capabilities could act in concert with a high glutamate diet to produce much higher concentrations of glutamic acid in an infant's blood than might be expected were such factors overlooked.

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Lowe and Zavon raise questions concerning specificity of glutamate-induced brain damage, doses, routes of administration, and absorption of glutamate from the gastrointestinal tract. We share their interest in these questions and in order to explore them (*1*, *2*) have conducted experiments in several hundred infant mice. Although not gathered on infant monkeys, these data may be of value in interpreting our infant monkey experiment (*3*) and in guiding future glutamate research with other preciously scarce primate infants.

In studies designed to investigate specificity and mechanism of action (*2*), mice (10 days old) were given (by feeding tube) chromatographically pure glutamic acid (*4*, *5*) (1 g/kg); this dose consistently induced a process of necrosis in hypothalamic neurons which was indistinguishable by electron microscopic examination from the neuronal pathology observed in the infant rhesus monkey treated with monosodium glutamate (*3*). Doses of sodium chloride as high as 8 g/kg (25 times the sodium content of monosodium glutamate at 1.0 g/kg) rendered infant mice nearly prostrate over a 4-hour observation period but resulted in no pathological alterations in hypothalamic or retinal neurons. Monosodium glutarate, which differs from monosodium glutamate only in that it has no amino group, induced no changes in hypothalamic or retinal neurons at 5 g/kg (*5*). The majority of amino acids tested induced no detectable changes in hypothalamic or retinal neurons at 3 g/kg (L-serine, L-glycine, L-alanine, DL-methionine, L-phenylalanine, L-proline, L-leucine, L-arginine, and L-lysine). Striking lesions did appear, however, in both the hypothalamus and inner retina after either oral or subcutaneous administration of sodium L-aspartate, sodium DL-α-aminoadipate, and L-cystiene. These findings suggest that the phenomenon of glutamate-induced brain damage to the immature central nervous system is not exclusively specific to glutamate but may be specific to a select group of compounds identified by others as having neuroexcitatory properties in vivo (*6*) and the ability to depolarize neural membranes in vitro (*7*).

When 10-day-old mice were fed monosodium glutamate in 10 percent aqueous solution by tube (*2*), hypothalamic damage occurred in 54 percent of 24 animals treated at doses of 0.5 g/kg and in 100 percent of 19 animals receiving 1.0 g/kg. In the same feeding experiments, a 0.5 g/kg dose of glutamate in combination with a 0.5 g/kg dose of aspartate resulted consistently in more severe hypothalamic damage than that induced by a 0.5 g/kg dose of either compound alone. Blood glutamate curves monitored in 23 tube-fed infant mice (*8*) indicate that oral intake of monosodium glutamate in doses of 0.5 to 1 g/kg produces high concentrations of glutamate in the blood, often exceeding 50 mg/100 ml by 15 to 30 minutes but returning to base-line values, in the range of 5 mg/100 ml, within 2 hours.

Lowe infers that 2.7 g/kg, the high dose of monosodium glutamate that we administered to test susceptibility in an infant monkey, represents an estimate

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of lowest effective dose. The only data on lowest effective dose that we are aware of are those for the mouse (*2*). Lowe's 20-jar margin of safety would become much narrower if he were to base his calculations on the lowest effective dose that we found in the infant mouse (0.5 g/kg). Lowe correctly points out that, until recently, some glutamate-rich strained baby food (4½ oz. jars) was supplemented with glutamate (615 mg) (*9*). In his calculation of risk, however, Lowe considered only the added glutamate. Moreover, it is uncertain, because unstudied, whether food with a high natural glutamate content (or glutamate plus aspartate content) imparts enhanced risk, and therefore calculations which ignore such fundamental considerations may not be valid. The weakness in Lowe's "obviously . . . no . . . risk" position, and in the position of others who have made similar pronouncements (*10*), is in the complete absence of supporting relevant experimental evidence. Glutamate was established years ago as a safe food additive by "expert" opinion and was introduced into baby foods without any safety tests being run on infant animals of any species. The question of safety would not have become an issue if experimental evidence rather than a priori assumption had been relied upon in the first place.

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23 December 1969

Brain Damage in Infant Mice following Oral Intake of Glutamate, Aspartate or Cysteine

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STRIKING degenerative changes in the infant mouse retina after subcutaneous treatment with monosodium glutamate (MSG) were reported by Lucas and Newhouse in 1957[1]. Other studies[2-6] established that the process of retinal degeneration induced by MSG treatment is a remarkably acute and irreversible form of neuronal pathology. Recently it was found that a similar process of acute neuronal necrosis occurs in several regions of the infant mouse brain after subcutaneous treatment with MSG, and that animals treated with high doses in infancy tend to manifest obesity and neuroendocrine disturbances as adults[7,8]. The arcuate nucleus of the hypothalamus is an area particularly vulnerable to glutamate induced damage in infant animals of several species (mice and rats[7], rabbits and chicks and the rhesus monkey[9]). In mice, which have been studied more extensively for MSG induced disturbances than other species, the infant animal suffered hypothalamic damage from a relatively low subcutaneous dose (0·5 g/kg of body weight)[7].

Table 1

Test compound	Dose (g/kg)	Number treated	Number affected	Necrotic hypothalamic neurones
Intubated, no treatment	—	10	0	0
MSG	0·25	10	0	0
MSG	0·50	23	12	7
MSG	0·75	16	13	13
MSG	1·00	19	19	25
MSG	2·00	7	7	40
L-Glutamic acid	1·00	4	4	23
Monosodium L-aspartate	1·00	4	4	26
L-Glutamate L-aspartate	0·50 0·50	8	8	27
Monosodium-glutarate	3·00	4	0	0
NaCl	3·00	4	0	0
L-Glycine	3·00	2	0	0
L-Serine	3·00	2	0	0
L-Alanine	3·00	2	0	0
L-Leucine	3·00	2	0	0
DL-Methionine	3·00	2	0	0
L-Phenylalanine	3·00	2	0	0
L-Proline	3·00	2	0	0
L-Lysine	3·00	2	0	0
L-Arginine	3·00	2	0	0
L-Cysteine	3·00	4	4	57

Each of the listed compounds was given in 10 per cent aqueous solution except L-glutamic acid, L-leucine, DL-methionine and L-phenylalanine which were given in 2·5 per cent aqueous solution because of their poor solubility in water. Because a large volume of fluid was needed to deliver high doses of L-leucine, DL-methionine and L-phenylalanine, only half the dose was given orally and the remainder subcutaneously. All of the other compounds were given orally. Sources of L-glutamic acid and MSG were purity checked by thin layer chromatography. Figures in the necrotic hypothalamic neurone column represent averages for each dose level.

Because of the widespread practice of weaning human infants on foods which are not only rich in natural glutamate content but may contain substantial quantities of glutamate (MSG) added for flavouring[10,11], it is important to establish whether damage to the infant central nervous system could follow from oral as well as from parenteral administration of glutamate[12]. We describe here experiments which demonstrate hypothalamic damage in infant mice following relatively low oral doses of glutamate, and also report that orally administered aspartate and cysteine can induce retinal and hypothalamic damage.

Seventy-five Webster Swiss albino mice, 10 to 12 days old, were given single oral doses of a 10 per cent aqueous solution of MSG at one of 5 dose levels (0·25, 0·5, 0·75, 1·0 or 2·0 g/kg). Ten control animals were intubated but given no treatment, and an additional 46 were given single oral doses of other test compounds, as shown in Table 1. Accurate dosage control was ensured by use of an improvised flexible gastric tube inserted gently through the mouth and oesophagus into the stomach. About 5 h after

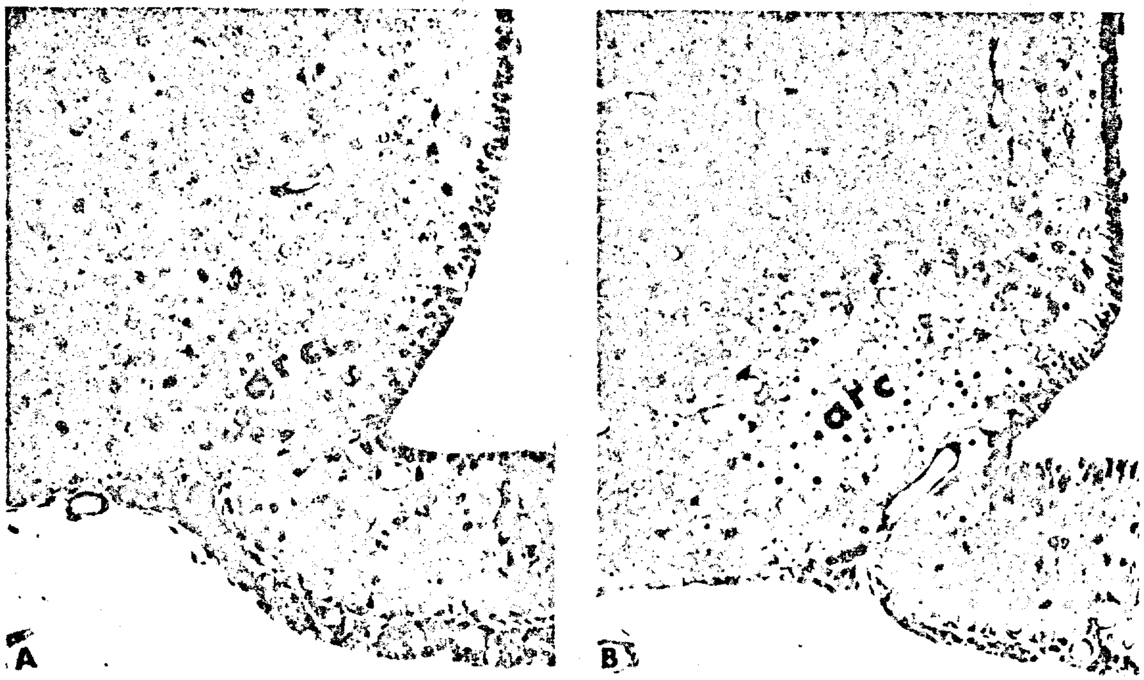

Fig. 1. *A*, Section through arcuate nucleus (arc) of hypothalamus from untreated 10 day old mouse. The tissue is well preserved by perfusion fixation and no signs of cytopathology are evident. Empty spaces are blood vessels dilated by perfusion fixation (×150). *B*, Section through arcuate nucleus (arc) from 10 day old mouse treated orally with MSG, 1 g/kg. There are approximately 33 necrotic cells in the arcuate region of this section (×150).

treatment, each animal was anaesthetized with chloral hydrate and killed by perfusion fixation of the central nervous system with 1·5 per cent glutaraldehyde and 1 per cent paraformaldehyde in 0·1 M cacodylate buffer. After 15 min of perfusion, the retinas and brain areas of interest were further fixed in osmium tetroxide and processed by a technique described elsewhere[9] which permits alternative examination of any specimen by either light or electron microscopy. To provide a rough estimate of the severity of brain damage at various dose levels, the hypothalamus of each animal was sectioned from its rostral (pre-optic) to caudal (post-infundibular) extent and necrotic neurones were counted in a representative section (1 μm thick) cutting across the arcuate nucleus at its level of maximal damage.

No evidence of cellular pathology was detected in the arcuate nuclei of any of the ten untreated control animals or in any of the ten animals treated with MSG at 0·25 g/kg (Table 1). Of the twenty-three animals given 0·5 g/kg doses of MSG, twelve (52 per cent) suffered hypothalamic damage; and of sixteen animals treated at 0·75 g/kg, thirteen (81 per cent) were affected. Nineteen animals (100 per cent) treated at 1 g/kg and seven (100 per cent) treated with 2 g/kg developed arcuate lesions. Necrotic neurone counts reflected considerable individual variability in response to MSG at any given dose level. But a comparison of average counts at each dose level revealed a consistent dose response relationship (Table 1). Brain sections from an untreated control animal and from an animal treated with MSG at 1 g/kg are illustrated in Fig. 1*A* and *B*.

We also found that a 1 g/kg dose of glutamic acid destroyed approximately the same number of hypothalamic neurones as a comparable dose of MSG, but neither sodium chloride nor sodium glutarate affected hypothalamic neurones at 3 g/kg. Most amino-acids tested (see Table 1) also failed to produce hypothalamic damage at 3 g/kg. Aspartate and cysteine, however, were striking exceptions because each animal treated with these compounds developed both retinal and hypothalamic lesions which seemed identical to those which are usually found after treatment with MSG. The possibility th glutamate and aspartate are additive in their toxic eff was suggested by the observation that every one of ea animals treated orally with a mixture of MSG (0·5 g/kg and sodium aspartate (0·5 g/kg) developed a degre hypothalamic damage characteristically seen in anim treated with either agent at 1 g/kg (Table 1).

Curtis[13] and others have found that glutamate, aspar and cysteine comprise a select group of amino-acids "neuroexcitatory" amino-acids) which can depola nerve membranes. Whether the striking ability of t select group of compounds to induce neuronal nec in the immature central nervous system relates to th ability to depolarize nerve membranes needs furt study.

Because glutamate is a naturally occurring constitu of dietary protein there has been little tendency to quest its safety for human infant consumption. But, in experiments, both glutamate and aspartate are toxic the infant mouse at relatively low levels of oral int and, when taken together, these common amino-ac have an additive brain damaging effect. Contrary conclusions which others have reached from studies adult animals[12] these experiments with tube fed inf animals raise serious questions concerning the advi bility of supplementing the human infant diet w MSG.

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Effect of Amino Acid Intake upon Serum Cholesterol in Man. ROBERT E. OLSON,* MILTON Z. NICHAMAN, JUDITH NITTKA, AND LAKELES DORMAN, Pittsburgh, Pa.

It has been shown that low protein diets (25 g per day) containing 36% of calories from relatively saturated fat induce a fall in serum cholesterol of 20% in human subjects (Olson and associates, Amer. J. clin. Nutr. 1958, **6**, 310). Supplementation of this diet with single amino acids produced no consistent response. The effect of various pure 1-amino acid mixtures was, therefore, studied to afford better control of the amino acid intake. For 4 weeks four male subjects were fed a control diet containing 100 g of protein, 36% of calories from fat, and all of the essential nutrients. Linoleate supplied 4% of total calories. Each subject was then given a formula diet containing either all of the 1-amino acids in the same proportions as in the control protein mixture or a diet containing the eight essential amino acids in adequate amounts plus glutamate as a source of nonessential nitrogen for 3 to 4 weeks. The caloric value and fat content of these diets were kept constant. The control diet was refed at the end of the experimental period. We found that when all the amino acids were fed, there was no significant change in serum lipids or beta-lipoproteins from control values. When eight essential amino acids plus glutamate were fed, however, serum cholesterol fell 37 mg per 100 ml, phosphatides 19 mg per 100 ml, and beta-lipoproteins 73 mg per 100 ml. Triglycerides actually increased 49 mg per 100 ml. All subjects remained in nitrogen balance during all experimental periods, showing that the hypolipidemic effect is not due to nitrogen imbalance. These data suggest that the formation of beta-lipoproteins by the liver is a function of the nonessential as well as the essential amino acid nitrogen in the diet.

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Oral and Subcutaneous Administration of Monosodium Glutamate to Infant Rodents and Dogs

REPORTS by Olney[1] and Olney and Sharpe[2] implicate monosodium glutamate (MSG) as a specific central nervous system toxicant in infant animals. Within a few hours, subcutaneous and oral doses of between 0.5 and 5 g/kg body weight produced intracellular oedema and neuronal necrosis in the paramedian plane bordering on the roof and floor of the third ventricle. Preoptic and arcuate nuclei of the hypothalamus were reported to be selectively affected, as well as the scattered neurones within the median eminence (nuclei tuberales). In mice given similar doses of MSG in infancy and allowed to grow to maturity, excessive weight gains were reported after 60 days. The Olney studies and the inference of potential risk from the use of monosodium glutamate as a seasoning agent have been the subject of polemical communications[3-5] and are under review by a committee of the National Academy of Sciences—National Research Council.

In comparative toxicological studies on rats (FDRL-Wistar derived), mice (C57Bl/6J), and beagles, we used appropriate controls with respect to both test materials and routes of administration. Except for the parent mice which were obtained from a commercial breeder, all animals were bred in the FDRL colony. Single doses were given either subcutaneously or orally at precise times after parturition to reproduce this phase of the Olney experiments. Solutions of MSG, monopotassium glutamate, sodium chloride, sodium gluconate, and distilled water were administered by both routes. Recognition was thus given to the role of both the sodium and glutamate moieties to the completely ionizable salt, sodium chloride, and to the less completely ionizable sodium gluconate. All doses were administered in 10% (w/v) aqueous solutions.

One series of mice and rats included 3 day old animals killed 24 h after single oral or subcutaneous doses administered between 1000 and 1200 h. Groups of five animals were used for each experimental treatment.

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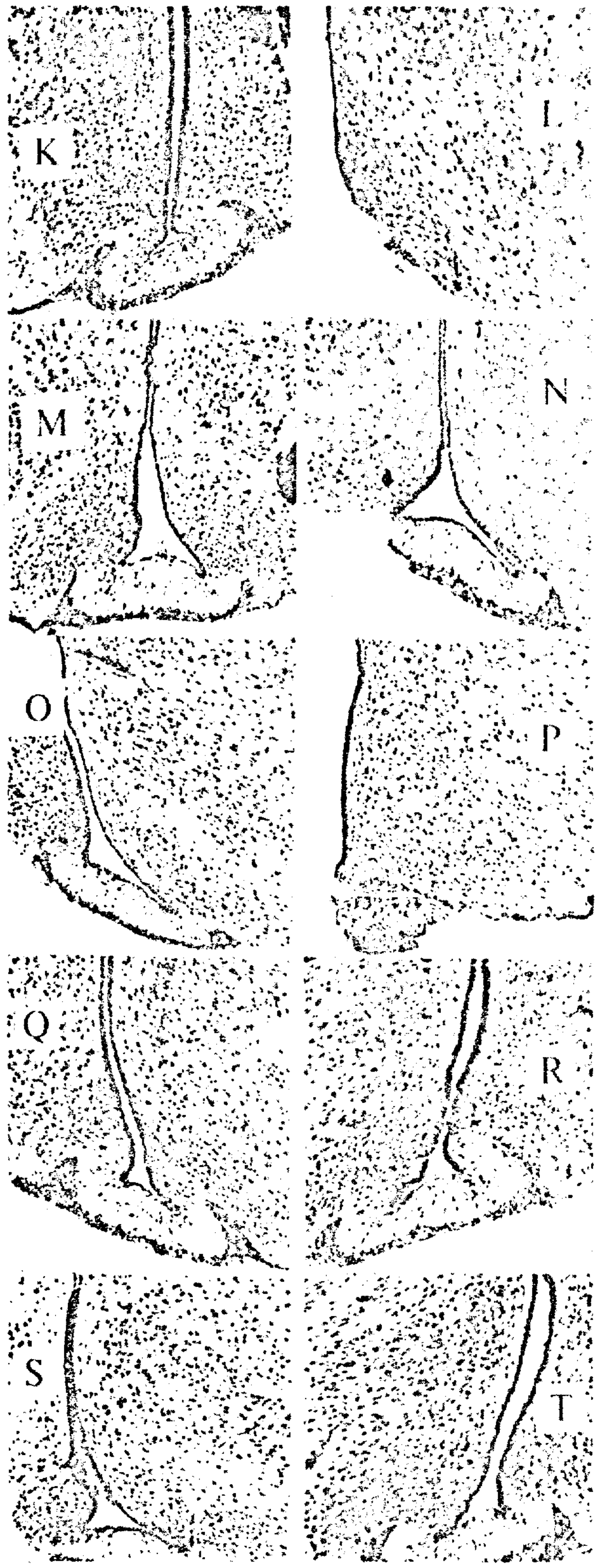

Fig. 1 Transverse (×60) sections, stained with haematoxylin and eosin through the hypothalamus at the level of the median eminence and arcuate nuclei. They represent 12 day old mice which received orally (left) or subcutaneously (right) monosodium glutamate (K and L), sodium chloride (M and N), sodium gluconate (O and P), monopotassium glutamate (Q and R) and water (S and T). These photomicrographs are characteristic of the findings in mice, rats and dogs at both levels (see text). The essential feature is the normal appearance and distribution of the neurones. T is an example of focal vacuolization of the ependymal cells, in 12 day old mice dosed subcutaneously with water. In K the arcuate nucleus has three small vacuolar spaces which, however, appear at higher power to be extracellular and may be artefacts. Similar changes can be found occasionally in almost all animals.

The age of the animals was taken into account inasmuch as MSG has been used as a food additive in baby foods. The period of introduction of "solid" foods into the diet of babies was reflected in a second series of rodents which were treated with a single dose at 12 days of age and autopsied 24 h later. At about 2 weeks of age, rats and mice begin to eat laboratory chow as well as taking maternal milk. The infant dog begins to ingest solid food after about 4 weeks and so for the beagles another series of experiments was started with single doses given at this time. The assignment of animals to treatment groups is shown in Table 1.

Mice and rats were autopsied after rapid anaesthetization with ether. Because neonatal and infant tissues are softer, it was possible to expose the brain, bisect it into right and left halves and remove it into fixative within 1–2 min. A further 1–2 min was needed to take multiple needle biopsies from the medial surface of the right half, through the areas of the pituitary base. These biopsy tissues and one eye were fixed in 3 per cent glutaraldehyde and subsequently stored in phosphate buffer at 4° C. The other eye was fixed in Zenker's fluid. The left half of the brain was fixed in 10% neutral buffered formalin, embedded in paraffin and 6 μm sections were cut from the region of interest. Transverse (coronal) sections, which best exhibit the target site, were taken from all but a few of the animals. Between twenty and forty sections were examined from most animals.

For preservation, dog brain was perfused intravascularly with 3% glutaraldehyde for 5–20 min. Needle biopsies from half or, more recently, thin slices through the floor of the third ventricle were removed and, together with retina and nerve of one eye, were further fixed in glutaraldehyde and stored in phosphate buffer at 4° C. For light microscopy, the standard haematoxylin–eosin staining procedure was used. A minimum of ten sections per animal was examined. Where lesions were found, the glutaraldehyde-fixed tissues were examined by electron microscopy.

Examination of the 3 and 12 day old rats and mice and of the 3 and 35 day old dogs revealed no significant differences among the test and control groups with respect to any of the treatment variables—the test solutions, the routes of administration or the age of the animals when dosed or killed. The slides were read by two pathologists, one of whom read the slides blind, and who agreed in their observations and interpretations.

The tissue changes observed in most of the brain section consisted of occasional small neurones with pyknosis or large cytoplasmic vacuoles and a sparse scattering of cells, presumably macrophagic or inflammatory, typified by distinct eosinophilic cytoplasm and variable numbers of nuclear lobes or nuclear fragments of various sizes. They were more apparent in 3 day old animals in which neuronolysis and neuronophagia were also found occasionally. The incidence of these changes, however, was not related to treatment nor were they selectively localized. They seemed to be related to the rapidity of growth of the neonatal brain. Fig. 1 is representative of the observations in the oral and subcutaneous studies for each of the five dosing variables of the 12 day old young receiving the 1 g/kg dose of their respective test materials.

Monosodium glutamate, monopotassium glutamate, sodium chloride, and sodium gluconate at 1 g/kg in a 10% w/v solution (and comparable volumes of distilled water) were administered orally and subcutaneously to mice and rats at 3 or 12 days of age and to dogs at 3 or 35 days of age and the animals were killed within 24 h of dosage.

Examination of the eyes and of the preoptic and arcuate nuclei of the hypothalamus by two pathologists revealed no dose-related histomorphological effects in any of the test groups at either of the two ages selected to correspond to the periods before and at the beginning of solid food intake. We offer no explanation for the fact that the observations here reported do not confirm those of Olney and of Olney and

Table 1 Assignment of Animals to Treatment Groups

Material	Mode of administration*	Mice 3 days 24 h	Mice 12 days 24 h	Rats 3 days 24 h	Rats 12 days 24 h	Dogs 3 days 3 h	Dogs 3 days 24 h	Dogs 35 days 3 h	Dogs 35 days 24 h
Monosodium	Oral	5	5	5	5	1	2	1	2
glutamate	Subcutaneous	5	5	5	5	1	2	1	2
Sodium	Oral	5	5	5	5	1	2	1	2
chloride	Subcutaneous	5	5	5	5	1	2	1	2
Sodium	Oral	5	5	5	5	1	2	1	2
gluconate	Subcutaneous	5	5	5	5	1	2	1	2
Potassium	Oral	5	5	5	5	1	2	1	2
glutamate	Subcutaneous	5	5	5	5	1	2	1	2
Water	Oral	5	5	5	5	1	2	1	2
	Subcutaneous	5	5	5	5	1	2	1	2

*Single doses of sterile 10% solutions of each (10 ml./kg body weight).

Sharpe after single doses of monosodium glutamate administered subcutaneously or orally to infant rats and mice.

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ELEVATION OF PLASMA GLUTAMATE IN GOUT*

Its Possible Role in the Pathogenesis of Hyperuricemia

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Abstract Fasting plasma glutamate concentration in 36 patients with gout was 65 ± 4 mμmoles per milliliter (± SE), and in 26 normal subjects it was 40 ± 3 (p less than 0.001). In 16 of the 36 patients fasting plasma glutamate was more than two standard deviations above the normal mean. After the ingestion of casein (0.5 gm per kilogram of body weight) plasma glutamate reached excessively high levels in the group with gout. Plasma glutamine and α-amino nitrogen were normal, both in the fasting state and after casein ingestion. The increase in glutamate may be causally related to the overproduction of purines in gout.

In a large proportion of patients afflicted with gout there is an increase in de novo purine production, which probably contributes importantly to the hyperuricemia in this disease.[1,2] The rate-determining step in the de novo synthesis of purines is the reaction of glutamine with 5-phosphoribosyl pyrophosphate to form 5-phosphoribosylamine (Fig. 1).[3] It has been suggested that the intracellular concentration of glutamine affects the rate of this reaction,

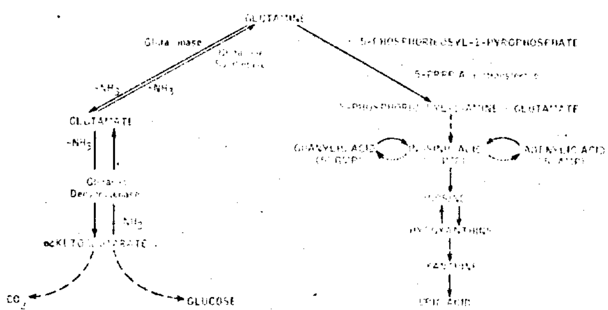

FIGURE 1. *Relation of Glutamine and Glutamate to the Biosynthesis of Purines.*

and that the enhanced purine production in gout may be secondary to an increase in this amino acid.[4] The fasting level of plasma glutamine is not higher in gout,[5] but this does not exclude the possibility of an increase in intracellular glutamine.

The consideration that intracellular glutamine may be elevated in gout has led us to investigate whether there might be an underlying increase in glutamate, because glutamate is the major precursor of glutamine.[6] In the present study we have measured plasma glutamate, glutamine and α-amino nitrogen in normal and gouty patients in the fasting state and after an oral casein load.

PATIENTS AND METHODS

Patients

The control group consisted of 26 male subjects who had normal serum uric acid and did not have a history suggestive of gout. Seventeen were ambulatory and healthy, and nine were hospital patients with a variety of illnesses.

The gouty group consisted of seven patients with asymptomatic hyperuricemia, and 29 who had had gouty arthritis and who had been found at some time to have hyperuricemia. Twenty-five of the patients with gout were ambulatory, and 11 were studied while hospitalized for a variety of illnesses. None had neoplastic disease, polycythemia or renal insufficiency. Twenty-two were taking allopurinol, probenecid or colchicine.

The mean age was 51 ± 2 years (±SE) in the control group and 51 ± 2 in the group with gout. Mean weight of the control group was 180 ± 6 pounds, as compared to 187 ± 5 in the patients with gout. Blood urea nitrogen and fasting blood sugar were within normal limits in all the control and gouty subjects.

Experimental Conditions

Venous-blood samples were obtained from both the hyperuricemic and control subjects between 8 and 9 a.m. after an overnight fast. No medications were taken on the morning of the study. Plasma glutamate content was determined on all the samples, and in some cases plasma glutamine and α-amino nitrogen were also measured.

In 18 gouty patients and 14 control subjects, after the initial blood sample had been drawn, 0.5 gm casein per kilogram of body weight was administered orally over 10 minutes. The caseinate† was given as a 15 per cent solution, to which sodium chloride in a concentration of 35 mEq/liter and a small amount of vanilla flavoring were added. Blood was obtained at 30, 60, 120 and 180 minutes for determination of plasma glutamate and, in some cases, glutamine and α-amino nitrogen.

All blood samples were placed in test tubes wetted with heparin, promptly immersed in ice water and centrifuged for 10 minutes in a refrigerated apparatus within 15 minutes after venipuncture. The plasma was then separated and kept at 4°C for a maximum of three hours. For determination of

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†Kindly provided as Casec by Mead Johnson Laboratories, Evansville, Ind.

glutamate, 1 ml of plasma was deproteinized by addition of 1 ml of 10 per cent trichloroacetic acid, the protein-free supernatant separated by centrifugation, and the supernatant stored at −20°C. Undeproteinized plasma samples for determination of glutamine and α-amino nitrogen were also stored at −20°C. All determinations were done within three days after the blood was drawn.

Plasma glutamate was determined in duplicate by a previously described modification[7] of the enzymatic fluorometric method of Graham et al.[8] Recovery of glutamate added to the plasma of gouty and control subjects was approximately 97 per cent. The glutamate content of plasma, as determined by this method, did not rise when the plasma glutamine was increased 500 mμmoles per milliliter by addition of glutamine, indicating that there was no marked generation of glutamate from glutamine during the course of the determination.

Plasma glutamine was determined in duplicate by the enzymatic method of Segal and Wyngaarden.[5] The plasma "ammonia blank" was determined by measurement of the ammonia liberated from plasma by addition of saturated potassium carbonate, and this amount was subtracted from the quantity of ammonia liberated by addition of saturated potassium carbonate after prior incubation of the plasma with glutaminase. Recovery of glutamine added to plasma of normal and hyperuricemic subjects was approximately 95 per cent.

Plasma α-amino nitrogen was determined in duplicate by the ninhydrin method of Fisher and his colleagues,[9] and serum uric acid by the uricase method of Liddle et al.[10]

Results

Mean fasting plasma glutamate in the 26 control subjects was 40 ± 3 mμmoles per milliliter (± SE), and in the 36 patients with gout it was 65 ± 4 (p less than 0.001). In 16 of the 36 gouty patients the plasma glutamate was more than 2 standard deviations above the mean of the normal group (Fig. 2). Mean plasma glutamate in the gouty patients taking allopurinol, probenecid or colchicine was not significantly different from that in the gouty patients who were untreated. In three patients studied both in the untreated state and after several weeks of therapy with allopurinol, 300 mg daily, no significant change in plasma glutamate was effected by the therapy (Fig. 2). Plasma glutamate did not vary with age or weight in the control or gouty subjects (Fig. 3). No significant correlation was noted between plasma glutamate and serum uric acid levels either in the control subjects or in the untreated hyperuricemic patients.

After ingestion of casein, plasma glutamate rose substantially in both control and gouty subjects, but was significantly higher in the gouty group at 30, 60, 120 and 180 minutes (Table 1). Nine of the gouty patients in the casein study had normal fasting plasma glutamate, and in three of the nine, the absolute rise in plasma glutamate after casein ingestion was significantly greater than that observed in the normal subjects.

Mean fasting plasma glutamine was 587 ± 30 mμmoles per milliliter in nine control subjects and 564 ± 17 in nine gouty patients (p greater than 0.1). In none of the patients was the glutamine level more than two standard deviations above the normal mean. Plasma glutamine did not rise excessively in the gouty patients after casein ingestion (Table 2).

Mean fasting plasma α-amino nitrogen was 2.72 ± 0.13 μmoles per milliliter in the control group and 2.57 ± 0.17 in the gouty group (p greater than 0.05). The levels of plasma α-amino nitrogen in the two groups did not differ significantly after casein ingestion (Table 3).

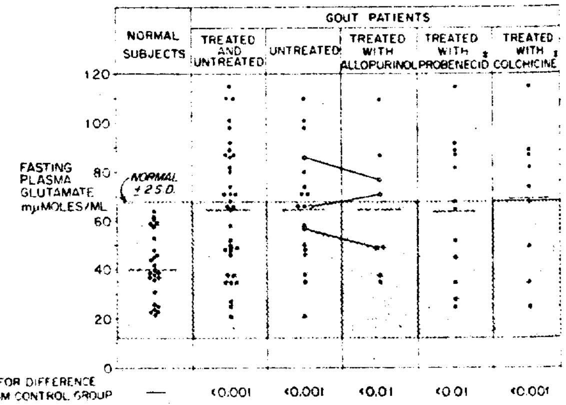

FIGURE 2. *Fasting Plasma Glutamate in Normal Subjects and Patients with Gout.*

Mean values for each group are indicated by the horizontal dotted lines. Open circles represent the plasma glutamate levels in three patients who were studied both in the untreated state and during therapy with allopurinol.

*Seven patients in this group were taking a combination of probenecid and colchicine.

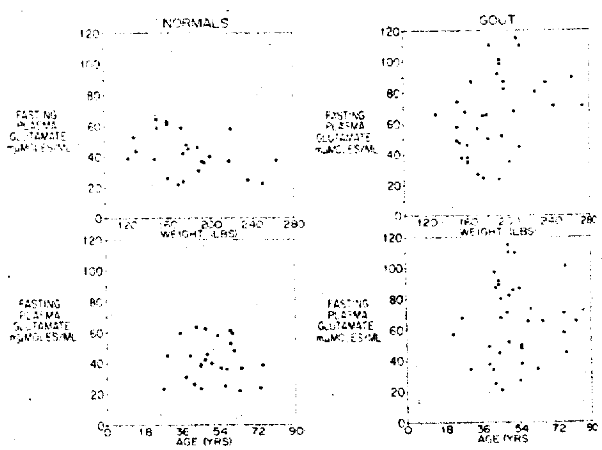

FIGURE 3. *Fasting Plasma Glutamate Levels in the Normal Subjects and in Patients with Gout.*

TABLE 1. *Plasma Glutamate Levels in Gouty Patients and Normal Subjects after Ingestion of Casein, 0.5 Gm per Kilogram of Body Weight.*

Subjects	Number	Plasma Glutamate (MμMoles/Ml ± SE)				
		at 0 time	at 30 min	at 60 min	at 120 min	at 180 min
Normal persons	14	45 ± 4	82 ± 6	80 ± 8	67 ± 9	56 ± 5
Gouty patients	18	68 ± 4	125 ± 10	126 ± 9	94 ± 8	83 ± 8
p value		<0.01	<0.01	<0.001	<0.001	<0.01

Discussion

In the present study it was observed that in about half the patients with primary gout, plasma glutamate was significantly elevated, suggesting the possibility of an increase in intracellular glutamate.

In view of the important role of glutamine in purine biosynthesis, it is pertinent to consider the possible relation between the elevation of glutamate and the overproduction of purines in gout. The rate-limiting step in the synthesis of purines is the reaction of glutamine and 5-phosphoribosyl pyrophosphate (PRPP) to form 5-phosphoribosylamine (Fig. 1),[3] and the intracellular concentration of glutamine may be one of the several factors that determine the rate of this reaction and thereby regulate the rate of purine synthesis.[4] Because glutamate is the major precursor of glutamine,[6] it is possible that in gout there is a primary defect in glutamate metabolism that causes accumulation of glutamate, resultant increase in cellular glutamine and consequent overproduction of purines. In addition to causing increased glutamine synthesis, a rise in glutamate would tend to promote elevation of glutamine by impairing its degradation, for glutamate is an inhibitor of glutaminase.[11] Inhibition of renal glutaminase due to the elevation of glutamate could also account for the apparent decrease in renal formation of ammonia in gout.[12]

Gutman and Yü[4] have postulated that the primary defect in gout may be a diminution in glutaminase activity, causing impairment of deamidation of glutamine to glutamate, and a resultant increase in the amount of glutamine available for reaction with PRPP. The elevation in glutamate observed in the present study cannot be explained by the hypothesis of Gutman and Yü, since a primary decrease in glutaminase activity would not increase formation of glutamate.

Plasma glutamine is not increased in gout, as demonstrated by Segal and Wyngaarden[5] and confirmed in the present study, but this does not exclude the possibility of an increase in intracellular glutamine. The concentration of this amino acid is normally several times greater in the intracellular than in the extracellular fluid,[13] indicating that the relation between intracellular and extracellular glutamine is not that of diffusion equilibrium. It is possible, therefore, that the intracellular concentration of glutamine can change substantially without an associated change in the extracellular level.*

The elevation of glutamate in gout is due almost certainly to an abnormality in the metabolism of this amino acid, rather than to impairment of urinary excretion, for in normal man only a minute fraction of ingested glutamate is excreted in the urine.[15] The elevation of glutamate in gout could be due to a decrease in activity of glutamic dehydrogenase (GDH), for in a person with a normal protein intake, the net flux of the GDH reaction is

TABLE 2. *Plasma Glutamine in Normal Subjects and Gouty Patients after Ingestion of Casein, 0.5 Gm/Kilogram of Body Weight.*

Subjects	Number	Plasma Glutamine (mμMoles/Ml ± SE)				
		at 0 time	at 30 min	at 60 min	at 120 min	at 180 min
Normal persons	9	587 ± 30	682 ± 35	715 ± 38	633 ± 26	581 ± 22
Gouty patients	9	564 ± 17	702 ± 36	677 ± 36	729 ± 39	686 ± 49
p value		>0.05	>0.05	>0.05	>0.05	>0.05

TABLE 3. *Plasma α-Amino Nitrogen in Normal Subjects and Gouty Patients after Ingestion of Casein, 0.5 Gm/Kilogram of Body Weight.*

Subjects	Number	α-Amino Nitrogen (μMoles/Ml ± SE)				
		at 0 time	at 30 min	at 60 min	at 120 min	at 180 min
Normal persons	9	2.72 ± 0.13	3.71 ± 0.20	3.66 ± 0.14	3.27 ± 0.15	2.97 ± 0.16
Gouty patients	9	2.57 ± 0.17	3.39 ± 0.31	3.37 ± 0.24	3.24 ± 0.07	2.77 ± 0.17
p value		>0.05	>0.05	>0.05	>0.05	>0.05

*The fact that the intracellular concentration of certain amino acids may be increased without an associated rise in the plasma level is exemplified in cystinosis. In this disease the free cystine content of leukocytes is 80 times normal, and yet plasma cystine is not increased.[14]

probably from glutamate to α-ketoglutarate, and a decrease in the activity of the enzyme would cause accumulation of glutamate. Interestingly enough, adenylic, guanylic and inosinic acids affect the activity of GDH in vitro.[16] Frieden[16] has suggested that the effects of these purine nucleotides on GDH may be involved in "feedback control" of purine biosynthesis, since alterations in GDH activity might affect the concentration of glutamate, thereby altering glutamine concentration and changing the rate of purine synthesis. It is of interest to speculate whether the primary defect in some patients with gout may be a decrease in GDH activity, owing to an abnormality in the interaction between the purine nucleotides and this enzyme. Clearly, this is not the basic defect in all patients with gout, since Kelley et al.[17] have demonstrated that in some patients the cause of the hyperuricemia appears to be a deficiency in hypoxanthine-guanine phosphoribosyl transferase.

In contrast with our observations, Kaplan et al.[18] observed normal levels of plasma glutamate in gout, but we believe that the method they employed for determination of glutamate was inaccurate. Using a chromatographic method, these authors found fasting plasma glutamate in normal males to be 180 ± 39 mμmoles per milliliter ($\pm$ SD), whereas with our specific enzymatic technic we find it to be 40 ± 14. It is unlikely that our result is artifactually low, for our mean recovery of glutamate added to plasma is 97 per cent. Furthermore, Stein and Moore,[19] using a chromatographic system in which the recovery of glutamate was 100 per cent, found mean serum glutamate in a group of normal males to be 50 ± 20 mμmoles per milliliter,[20] a value similar to ours. As we have elaborated elsewhere, the higher values obtained by Kaplan and his colleagues[18] are probably due to generation of glutamate from glutamine during storage of the plasma or during chromatography.[21]

Kaplan et al.[18] have demonstrated convincingly that in gout there is an increase in the plasma concentration of at least nine amino acids other than glutamate (aspartate, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine and lysine). In view of this observation, the finding in the present study that plasma α-amino nitrogen is not elevated in gout is surprising and unexplained. Of the nine amino acids found to be increased, the elevation of all but lysine may be secondary to the increase in glutamate, for all except lysine are in equilibrium with glutamate through transamination reactions.[22]

We are indebted to Miss Elizabeth Sheldon and Miss Judith Igler for technical assistance and to Dr. Alton L. Steiner for collaboration during the initial phase of this work.

Since submission of this manuscript for publication, Yü et al.[23] have reported that in gouty patients, plasma glutamate is elevated, and plasma glutamine and total plasma amino acids are normal, as measured chromatographically. Their findings are similar to those reported in the present paper.

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PROTEIN HYDROLYSATES
AND FLAVOR ENHANCERS

A paper given October 29, 1970, by Mr. H. Pease to the graduate course in Food Product Development at Columbia University.

PROTEIN HYDROLYSATES AND FLAVOR ENHANCERS

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FOOD PRODUCT DEVELOPMENT - NUTRITION P-6213

PROTEIN HYDROLYSATES AND FLAVOR ENHANCERS

I. Food Ingredients Used to Improve and Intensify Flavor

Since prehistoric times man has used ingredients, now we call them additives, enhancers, or potentiators, to improve and to intensify the flavor of foods. Whether these ingredients were salt, sugar, or spices such as pepper, cloves, nutmeg, turmeric or cinnamon, they were essential to the preparation of flavorful food. We would like to discuss the utilization of three ingredients* - hydrolyzed plant protein, monosodium glutamate and nucleotides as additives to present day foods.

The three terms, seasoning, enhancer and potentiator, are widely used to describe such ingredients. We like to define them as follows:

A. A seasoning is a substance which provides a characteristic flavor of its own such as, salt, spices, sugar, acids, HPP.

B. An enhancer strengthens and improves flavor inherent in a product without adding a flavor of its own. MSG added to chicken soup improves the chicken flavor without adding its own flavor.

*Abbreviations to be used in this paper are -

HPP - Hydrolyzed Plant Protein
MSG - Monosodium Glutamate
5'IMP & 5'GMP - Disodium Inosinate & Disodium Guanylate (Nucleotides)

C. The term, flavor potentiator, was coined by Arthur D. Little, Inc for a flavor symposium held May 18, 1964. Potentiation had been a term used in pharmacology to describe an action wherein the agent, by itself in small quantities, has no effect on the biological system but exaggerates the effects of other agents in the system.

Flavor potentiators give a synergistic effect so that the whole is greater than the sum of its parts. Two ingredients interact to produce an effect not attainable by either alone. All three ingredients, nucleotides, MSG and HPP can act as potentiators.

D. In many use applications, there may not be a clear distinction as to whether an ingredient is acting as a seasoning, an enhancer or a potentiator. There is often an overlapping effect which makes an exact definition impossible. In most cases, the type of action is dependent upon use level in the flavor system. Salt, when used at a high level, is a seasoning, and at a low level of use, becomes an enhancer. MSG, when used alone, is an enhancer; but when used in combination with nucleotides, is a potentiator. From our experience, hydrolyzed plant protein may fall into any one of the three categories depending upon its use level and the flavoring system.

II. Hydrolyzed Plant Proteins

A. Definition

The term hydrolyzed plant protein implies the conversion of proteins into amino acids. Proteins, which are polymers of amino acids, consist of a group of amino acids held together by a peptide linkage between an amino group and an acid group. In preparing protein hydrolysates, this peptide linkage is broken by introducing water through the use of a catalyst (acid, enzyme or alkali). The resulting breakdown products, which are amino acids or salts of amino acids, are soluble and have a wide flavor spectrum. Data sheet #32 shows the typical chemical structure of a protein and an amino acid.

B. Historical Background

Protein hydrolysates have been known and used for flavoring foods for many years. Soy sauce, widely used in China and Japan for adding flavor to their bland rice and vegetable dishes, is produced from an enzymatic hydrolysis of soy protein. Maggi Seasoning, introduced in Switzerland in 1886, is an acid hydrolysis of plant protein. It gained wide acceptance throughout Europe as a flavoring aid. Meat extracts and yeast extracts are other protein hydrolysates which have been used for years.

C. Two types of protein hydrolysates are commercially available

1. Acid hydrolysates are made from plant or vegetable protein.

2. Enzymatic hydrolysates are made either from Baker's Primary yeast or Brewer's yeast, and commonly sold as yeast extracts or yeast autolysates. The products made from Brewer's yeast usually have a rather bitter flavor accent.

D. Manufacture of Hydrolyzed Plant Protein

1. Processing stages and a flow diagram for a typical HPP are given on data sheets #33 and #34. The procedures involve hydrolysis, neutralization, filtration, and concentration. Individual manufacturers have special treatments which give identity to different products.

2. Methods for Modifying Flavor

Hydrolyzed plant proteins, having widely different flavor accents and color characteristics, can be produced by modifying such processing aspects as protein blend, hydrolysis conditions, neutralization end point, concentration, decolorization treatment, and enrichment, as well as by employing special processing techniques usually considered trade secrets.

3. Physical Characteristics

HPP is available in three forms: liquid, paste and powder.

The powder form is hygroscopic and for ideal handling conditions, the relative humidity should be under 30%. Manufacturers are now producing HPP with improved handling properties through use of such techniques as oil coating, increasing the particle size, or using flow conditioners.

HPP powder will usually fuse at temperatures much above 95° F. It is therefore essential that HPP be stored at temperatures under 95° F. and that it be packaged in moisture protective containers.

4. Price Structure

The price of HPP varies with the type of grade, but the present price range is about 60¢ a pound.

E. Grades Available

1. Acid Hydrolysates can be classified into four grades: by-product, high quality, special blends and reaction products.

a. By-product HPP with a portion of the MSG removed.

This was the first type of HPP available in the United States. It usually had a harsh, bitter flavor often described as a 'rubber boot taste'.

b. <u>High quality HPP</u>

By 1959, HPP's, which were completely different from the old style by-product HPP, were being marketed. These HPP's were both flavor contributors in their own right and enhancers of inherent flavor of meat, poultry and seafood. The products had a clean, sweet, meat-like flavor that found ready applications in a wide variety of foods. They lacked the objectionable harsh, bitter taste of the old style hydrolysates. Although the HPP's now available do vary greatly in flavor characteristics and quality, a major portion of HPP sales are in the so-called high quality lines.

c. <u>Special HPP Blends</u>

More and more, manufacturers are making available blends of HPP with such other flavor potentiators as MSG and nucleotides. One of the best HPP blends now available contains a mixture of HPP, yeast extract, MSG, and nucleotides. There is a very popular HPP containing an added smoke flavor.

Because of its outstanding enhancing properties, HPP is an ideal carrier for many flavors.

d. HPP Reaction Products

Three patents issued to Lever Bros. in April, 1960 described the production of products having a cooked or roasted meat flavor. The items were produced by heating sulphur containing amino acids (methionine, cysteine) and five carbon sugars (xylose, ribose). Patents on other so-called reaction products have since been issued to Pfizer, I.F.F. and Nestlé. In most cases, the products have a reaction flavor superimposed on HPP fortified with MSG and nucleotides.

2. Yeast Autolysates

Yeast autolysates normally are available in such products as standard, low sodium and special blends fortified with MSG or nucleotides. Each grade is usually available in a dark or a light color. Data sheet #37 gives a typical analytical composition of yeast extract.

F. Composition and FDA Status

1. HPP consists of a blend of amino acids and salts of amino acids. Sodium chloride is a naturally occurring ingredient since it is produced during the neutrali-

zation operation. HPP is normally sold as a flavoring material, and no nutritional claims are made. During the acid hydrolysis tryptophane is destroyed, and therefore all the essential amino acids are not present unless they are added. Data sheets#35 and#36 give typical chemical and amino acid compositions of HPP.

2. FDA Status

Hydrolyzed plant proteins are listed as GRAS (generally regarded as safe) ingredients, and no limitation is placed on their level of use. The use levels may vary up to 1.5% as consumed, dependent upon the type of product and whether HPP is used as a seasoning, enhancer or potentiator.

G. Flavor Characteristics and Utilization

1. HPP has all the basic flavor characteristics - sweetness, saltiness, bitterness and sourness. Hydrolyzed plant proteins have meat-like flavors which cover a wide spectrum. They are being used successfully in beef, pork, veal, poultry and fish dishes. In addition, HPP is widely used to intensify, blend, or suppress other flavors in practically all food areas, canned, frozen, or

dehydrated whether the foods are soups, sauces, vegetables, salad dressings, casseroles, meats, or snacks. Some of the best uses for HPP are:

a. To intensify and impart meat-like flavor in soups and sauces.

b. To replace meat or beef extract.

c. To impart a pleasing flavor profile.

d. To add flavor to main course dishes containing rice, potatoes, vegetables.'

e. To replace MSG.

f. To act as a sparing agent for nucleotides.

2. Certain precautions must be taken when formulating with HPP. Since it is hygroscopic, HPP when used in dehydrated mixes, must be combined only with ingredients having low moisture content; otherwise caking will result. Combinations of HPP with reducing sugars will often result in a Maillard browning reaction. Dehydrated mixes containing HPP must be packaged in a moisture protective unit to prevent caking.

III. Monosodium Glutamate

A. Historical Background

Monosodium glutamate is the sodium salt of glutamic acid.

Glutamic acid was first isolated in 1866 by a German chemist, Ritthausen; however, it was not until 1908 that its flavor building properties were discovered by Dr. Ikeda of Tokyo University. He found MSG to be the important flavor component of sea tangle, an edible seaweed which was being ground and used to flavor Oriental dishes. Within a decade commercial production of MSG was begun, and by 1933, Japan was making ten million pounds a year. Rapid commercial adaptation was possible because processing required only the acid hydrolysis of protein (wheat gluten), a technique which was well known at that time. The first United States plant was started by Huron Milling in 1935, and by the early 40's there were at least three other manufacturers in the United States (International Minerals, General Mills, A. E. Staley). Raw material sources used were wheat gluten, corn gluten, beet sugar, and Steffens waste. Approximately forty million pounds of MSG were used last year in the United States and two hundred fifty million world wide.

B. Manufacture

1. Acid Hydrolysis

The classical method of manufacture of MSG is by extraction from natural sources such as vegetable proteins and sugar beets. One method employs the acid hydrolysis of wheat gluten, the separation of the glutamic acid, its purification, and conversion to the sodium salt followed by the processes of crystallization and drying. This method yields a high proportion of by-products - eleven pounds of starch are obtained for each pound of glutamic acid and a residual mixture of amino acids which are often used for soy sauce or low quality HPP.

2. Fermentation

A fermentation process is now utilized almost exclusively for the production of MSG. Common raw materials are simple sugars such as glucose obtained by hydrolysis of starches. The fermentation is carried out by certain microorganisms in a culture medium containing a source of nitrogen (NH_3), growth factors and small amounts of inorganic salts. Since 1959, when the fermentation process began to be used, prices of MSG have declined from approximately $1.50 per pound to 40¢ per pound.

3. Chemical Synthesis

Several synthetic processes have been developed for the manufacture of MSG. Instead of doing a fermentation or chemical extraction of natural products such as wheat, corn, beet sugar, soy beans, Ajinomoto, world's largest producer of MSG has developed a process for producing MSG from a petro chemical. The reason for their interest in a chemical synthesis is that the old extraction process turns out large quantities of by-products - starches and amino acids to be disposed of. As the market for MSG increased, the markets for the other items became saturated.

C. Composition

1. Chemical and physical characteristics

MSG is the sodium salt of glutamic acid. It is a free flowing, non-hygroscopic, dust free crystal which is usually offered in several particle sizes, in grades having 99% purity. MSG is very stable under heat processing conditions.

D. Flavor Characteristics and Utilization

1. When tasted in water, MSG has a sweet, saline taste accompanied by some astringency. It can be used to intensify a wide variety of foods without adding any flavor of its own. Theories to explain its flavor enhancing properties have speculated that MSG stimulates the taste buds and therefore increase the response to a flavor or that it stimulates saliva formation. It is particularly good for developing chicken flavor and is widely used in homes as an all purpose seasoning agent sold under the Accent brand name. The use levels usually vary from 0.1% to 0.5% as consumed.

2. MSG is best used in a pH range of from 5 to 7. It is not effective in the low pH range.

3. It is reported that MSG has a preservative action on flavor and color although we have not encountered such an activity in our work.

E. <u>FDA Status</u>

1. MSG is listed as a GRAS ingredient, and there are no limitations on levels of use; however, recently questions have been raised as to its safety.

2. <u>Chinese Restaurant Syndrome</u>

In July of 1968, an article in the <u>New England Journal of Medicine</u> proposed MSG as being the cause of 'Chinese restaurant syndrome' in which individuals have reported headaches after having eaten Chinese meals. The February 21, 1969 issue of <u>Science</u> carried an article in which tests indicated there were three categories of symptoms elicited by MSG - burning sensation, facial pressure, and chest pains. Headaches were a constant complaint in a majority of the individuals. These symptoms appeared only if the meal was taken on an empty stomach by a susceptible individual.

MSG has been used since the early 1900's as a food enhancer. Its per capita consumption in the United States is estimated at a maximum 1.2 grams per day. Orientals, for many years, have been heavy consumers of MSG with an estimated average of 6 grams per day. Scientific literature from these countries record no evidence of ill effects.

International Minerals and Chemical Corporation, in a statement to the United States Senate Committee on Nutrition and Human Needs, cited a two year chronic feeding study in rats made in 1950-51 by Arthur D. Little in which rats were fed MSG up to 0.4% of the weight of the diet with no ill effects and a two year study on mice which were fed a diet consisting of levels of MSG equal to 4% of the weight of the diet (equivalent to one pound of MSG a day for a man weighing 150 lbs.). The scientists were looking primarily for possible tumors in these animals. There were none nor were there any toxic effects.

In a more recent development concerning the use of MSG as a food additive, Dr. John Olney, a Washington University brain researcher, told in 1969, about injecting small amounts of MSG into the veins of baby mice, examining their brains through an electronic microscope, and detecting shattered and pitted cells in certain brain areas. Detailed studies of what MSG would do to the brain cells of very young animals had never been done before. Not only has he found damage in mice but also in monkeys. In later work, Olney reported seeing eye and brain damage in infant mice when MSG was taken orally. He holds that on a weight per weight basis, the amount of MSG he uses to produce brain damage in mice is only five to six times the equivalent amount a baby receives by eating a complete jar of high protein baby food, and many babies eat two. He also pointed out that MSG serves no real purpose for babies and that it is put into baby food to enhance the flavor to the satisfaction of the mother's pallet, not the infant's. "Babies' taste buds are too underdeveloped to detect much of a meat taste", he says. As a result of this publicity, baby food manufacturers in the fall of 1969 voluntarily discontinued using MSG as an ingredient. It is ironical but in a paper given on "The Pharmacology of Glutamic Acid" during a symposium on MSG in 1948, it was reported that glutamic acid increased I.Q. and produced beneficial effects in the behavior of mentally retarded children.

The report of the National Academy of Sciences - National Research Council Committee issued in July, 1970 and covered by an FDA news release dated August 7, 1970, apparently settles the question of MSG for the time being. The Committee concluded that the risk associated with using MSG in foods for infants is extremely small. The Committee cannot find, however, that the usage confers <u>any</u> benefit to the child and therefore recommends that MSG not be added to foods specifically designated for infants.

The Committee found no evidence of hazard from the reasonable use of MSG in foods for older children and adults, except for those who are individually sensitive to the substance. The flavor-enhancing property of MSG is considered to be beneficial to the general consumer in these age groups. The Committee therefore recommends that use of MSG be permitted in processed foods for these groups and that such foods be clearly labeled to indicate the presence of added MSG for the information of those who wish to avoid it. Sale of MSG in packages for institutional and home consumer use need not be curtailed.

IV. Nucleotides - New Flavor Potentiators

A. Historical Background

Inosinic acid was first isolated from meat extract in 1847 by Liebig. Over fifty years later, in 1913, Kodama first identifie the primary flavor component of dried bonito as being the histidine salt of inosinic acid. In contrast to MSG, there was not available the necessary technical knowledge for the commercial production at that time since the biochemistry of nucleic acid is a more modern field than that of protein or carbohydrates.

In 1951, Dr. Kuninaka undertook a study of the enzymatic degradation of nucleic acid by microorganisms including a thorough study of the flavor characteristics of each degradation product. By 1957 and 1958, the following facts became known:

1. Among the three isomers of IMP, only the 5' IMP has flavor activity.
2. Histidine is not necessary for the flavoring action of 5' IMP.
3. 5' GMP also has flavoring activity similar to the 5' IMP.
4. There is a synergistic effect between MSG and 5' nucleotides.
5. The enzyme was identified to produce 5' nucleotides from ribonucleic acid (RNA).

B. Manufacture

The 5' nucleotides, first offered commercially in Japan by Takeda in 1959, were made by the enzymatic degradation of yeast nucleic acid. They are currently being produced by three companies in Japan - Takeda, Yamasa and Ajinomoto.

C. Types

The two commercially available nucleotides are disodium inosinate (5' IMP) and disodium guanylate (5' GMP). These are available as free flowing crystals either singly or in blends. The most popular blend is one containing 50% 5' IMP and 50% 5' GMP sold under such brand names as Ribotide, Mertaste and IG. We have found that 5' IMP and 5' GMP give the same flavor effects but that they differ in strength. In our work, we have found the disodium guanylate to be three times as effective as disodium inosinate. The common blends of 50% of each have a flavor strength about double that of disodium inosinate.

When nucleotides were first commercially available in the United States, approximately 1962, the price was $50.00 per pound. At the present time, the price is $6.50 per pound for a blend of 50% 5' IMP and 50% 5' GMP.

D. Flavor Characteristics

1. Nucleotides have a sweet, meaty flavor note characteristic of all meat products but not associated with any particular variety. Since nucleotides are found in nearly all meats, this aspect might be expected.

2. Nucleotides give a bodying effect - impart mouthfeel.

3. Nucleotides have a synergistic effect with MSG and HPP. The MSG sparing effect has been reported often in the literature. It has been said that 0.2% of MSG in dehydrated soup mixes can be replaced with 0.1% MSG and 0.001% nucleotides (50% sparing) or by 0.05% MSG and 0.002% nucleotides (75% sparing).

4. Nucleotides suppress some flavors - bitterness, sulphury notes and sourness. Ingredients usually not effected are butter, fat and spices.

5. Nucleotides give an MSG effect - saline taste with a mouth filling effect and astringency aftertaste but they are much sweeter and meatier than MSG.

6. Nucleotides are used at very low levels, usually from 25 to 100 ppm as consumed.

E. <u>Utilization</u>

From our experience, we believe nucleotides are best used in combination with HPP or MSG. There is a definite synergistic effect when used with these ingredients. Some of the best use applications are:

1. To strengthen meat flavors in soups and gravies.

2. To replace meat extract. A few years ago, meat extract was widely used for flavoring and it has now been largely replaced by using blends of HPP and nucleotides.

3. To suppress undesirable flavors.
4. Some decomposition is obtained in heat processing (approximately 10% when heated at 240° F. for 1 hr.) and degradation can occur when utilized in systems containing certain enzymes (phosphatase).

F. <u>FDA Status</u>

Nucleotides are approved by FDA for use as food additives. This is not surprising since nucleotides are found in a wide variety of food materials such as yeast, chicken, beef and pork. The levels range from 0.2% to 0.8% in animal tissue to about 1.2% in Baker's yeast.

Usage levels approved for flavor modification or enhancing meats, poultry, vegetables, soups, sauces, gravies, and cheese dishes were up to 0.1% for disodium inosinate and up to 0.06% for disodium guanylate.

The FDA approval stated that "our pharmacologists conclude that the usage of disodium inosinate or disodium guanylate as flavor enhancers in food is safe primarily because they are normal constituents of man's diet and the proposed use levels are lower than the levels of natural occurring inosinic acid or guanylic acid in some foods. The biochemical studies and the toxicity studies provided additional assurances that these products are safe for usage in foods".

V. Synergistic Effects of Flavor Enhancers

We have discussed the flavor characteristics and utilization levels of HPP, MSG and nucleotides when used individually. Our experience has been that the best results can be obtained by using combinations of HPP, MSG, and nucleotides.

A. Hydrolyzed Plant Proteins and Monosodium Glutamate

The addition of MSG to HPP will often strengthen and intensify the inherent flavor of the HPP. Such blends may allow lower use levels or provide a more desirable flavor release. These combinations will not usually change the basic flavor character.

B. Monosodium Glutamate and Nucleotides

There has been much in literature to point out the synergism between MSG and nucleotides as well as an MSG sparing effect. Actually, this synergistic effect led to the discovery of the flavor potentiator possibility of nucleotides since the latent flavor level of IMP and GMP was markedly detectable when evaluated in an MSG solution. In water, a threshold level of 0.01% 5' IMP was found, but in the presence of 0.1% MSG, the flavor effect was almost equivalent to that of 0.5% MSG. Synergistic action was also demonstrated by studying the mutual effects in reducing individual threshold levels; for example, the threshold level of nucleotides is reduced sharply in MSG solution and the threshold level of MSG is reduced sharply in a nucleotide solution. The flavor action of a 0.3% MSG solution is equivalent to that of a 0.087% solution of a 20 to 1 mixture of MSG and 5' IMP; in other words, the flavor activity of the mixture is about 3.4 times as much as MSG.

C. <u>Hydrolyzed Plant Protein and Nucleotides</u>

Our test kitchen trials and panel tests have indicated that there is a remarkably effective synergism between HPP and nucleotides that yields flavor systems superior to any available for use in such foods as soups, gravies, sauces, vegetables and meats. The threshold level of Ribotide was found to be 0.013% and that of HPP to be 0.024%; but when a blend of 50% of each was used, the threshold level became 0.006%. When evaluated in either a beef style broth or a chicken style broth, a blend of 0.02% HPP and 0.08% Ribotide was preferred overwhelmingly over a 0.1% level of Ribotide. Our study indicates that

HPP has a great nucleotide sparing effect. A blend of 95% HPP and 5% nucleotide is often as effective in its flavor enhancing properties as a blend of 95% nucleotide and 5% HPP. Since such a blend would cost only 13% that of a blend containing the higher level of nucleotides, this synergism offers potential for considerable raw material cost savings in formulations.

VI. FORMULATING WITH PROTEIN HYDROLYSATES AND FLAVOR ENHANCERS

A. Areas for Utilization

Protein hydrolysates and flavor enhancers are now being used in a wide variety of foods - broths, bouillons, soups, stews, gravies, sauces, meats, dips, salad dressings, casseroles, main course dishes with potato, rice or pasta, snacks, seasoning mixes, breading mixes, fish and many other foods. In fact, new uses are probably being found daily as more food technologists are evaluating the use of protein hydrolysates. All types of foods - dehydrated, frozen, heat processed or refrigerated are included in these applications.

B. Procedures for Designing the Composition of a New Product

1. Survey of Competitive Products

After the product concept has been established, our development work usually begins with a survey of competitive products if the concept is not entirely new and comparable products are commercially available.

a. The analytical composition is established by doing simple determinations of total solids, ash, sodium chloride, nitrogen and fat. From this information, an estimate is made of the content of moisture, minerals, sodium chloride, organic solids, fat, protein and carbohydrate. For example, a dehydrated chicken flavored broth might give the following analytical composition:

Total Solids	98.0%
Ash	48.0%
Chloride, calc. as NaCl	45.0%
Total Nitrogen	2.4%
Fat (Ether Extract)	7.5%

b. Ingredient Composition

From the above analysis, the following estimate can be made of ingredient composition:

Moisture Content	2.0%
Minerals	48.0%
Organic Solids	50.0%
Protein (Nx6.25)	15.0%
Fat	7.5%
Carbohydrate (by difference)	27.5%

This information, together with that declared on the ingredient list, can be transmitted into the following crude ingredient approximation:

HPP and Flavor Enhancers (derived from protein content - HPP may vary from 30% to 40% protein)	35% to 50%
Sodium chloride	45%
Sugar and Starch	27%
Flavorings (onion, celery, garlic, turmeric and parsley)	6%
Fat	7%

c. Such aspects as flavor, texture, processing, directions for use, packaging, and shelf stability are prime considerations in formulating a food product. In a meat flavored broth, the meaty accent, sweetness, bitterness and sourness should be carefully evaluated to help in the selection of the best flavor profile. Textural aspects are very important in some foods especially gravies or sauces. Even in a broth, we are concerned about mouthfeel, hoping to obtain a full bodied broth. Processing procedures are of concern and can greatly affect flavor in the final product. In this instance, the broth could be made by wet processing or by merely blending the dry ingredients. Directions for use can be critical in some items and adequate test kitchen trials must be made to be certain that customer directions are foolproof and adequate. The type of package should be dictated not only by Marketing desires but also by the nature of the product, whether it is hygroscopic and needs protection against moisture pickup or whether it is sensitive to light. Of course, accelerated storage and shelf stability tests are an essential phase of any product development program.

2. Preparation of a Prototype Sample

After obtaining preliminary information on approximate ingredient composition, the actual work on formulating begins. Since the flavor is of main interest to us, we will review the method we use to evaluate HPP and flavor enhancers in a dehydrated broth formulation. From the approximate analytical composition, we calculate the composition for an individual portion. For the chicken flavored broth, the composition in grams for a 6 oz. portion might be as follows:

Salt	1.75 grams
Sugar	0.55 "
Starch	0.581 "
White Pepper	0.005 "
Turmeric	0.005 "
Onion Powder	0.100 "
Celery Salt	0.100 "
Garlic Powder	0.004 "
Parsley	0.005 "
Chicken Fat	0.300 "

A base would be made up with this composition after which individual 3 gram/6 oz. portions would be prepared for evaluating the level of use of HPP and flavor enhancers. Starting levels of use for the product as consumed might be as follows:

HPP	0.5%
MSG	0.2%
Nucleotides	0.01%

Since the basic flavor characteristic is usually imparted by the HPP, we first determine the best level of use for HPP after which MSG or nucleotides may be added to determine if an improved flavor characteristic can be obtained. As an example of the flavor possibilities obtained by adding HPP, we have prepared this sample formula for a broth. We would like you to taste it as is and with added HPP which, in this instance, happens to be Maggi Super 3H. Actually, Maggi Super 3H is a blend of the flavor enhancers HPP, MSG and nucleotides.

For evaluating HPP in a chicken gravy, we could take the same broth base to which is added 5 grams of nonfat milk solids, 4 grams of flour and 3.5 grams of starch to a 6 oz. portion. To obtain an acceptable flavor strength in the gravy, the levels of HPP, fat and flavorings will need to be increased considerably. For evaluating the use of HPP in a chicken noodle soup, this same broth base could be used with 5 grams of added noodles to each 6 oz. portion. Here again, the flavor levels may need to be increased over those determined for the broth.

As with most ingredients, there are precautions which must be taken when formulating with HPP. Since HPP is hygroscopic, it will tend to pick up moisture either from the air or from other ingredients. If the pickup is excessive, the product will cake or fuse. Because of this tendency, packages for dehydrated products containing HPP should provide good moisture protection and the formula should not use any ingredients having high moisture contents (i.e., undried starch). Under the right conditions of moisture and heat, hydrolyzed plant protein and reducing sugars will combine in a Maillard browning reaction. The use of corn syrup solids and dextrose with HPP should be checked carefully in an accelerated storage test program.

VII. New Concepts for Flavor Improvement

In summary, the three food ingredients HPP, MSG, and nucleotides play an important part as enhancers and potentiators in present day foods. Optimum results can usually be obtained by using combinations of the three. Because of the complex nature of the synergistic effects of the three ingredients, best results can usually be obtained by using one of the commercial blends now available. It is very probable that HPP, MSG, and nucleotides are only the forerunners of new potentiators that are now being developed by food scientists.

In the controversial atmosphere now surrounding food additives, most food processors undoubtedly will take Alexander Pope's advice "Be not the first by whom the new are tried nor yet the last to lay the old aside".

We still believe that the three ingredients, hydrolyzed plant protein, monosodium glutamate, and nucleotides, all natural derivatives of foods, will continue to receive FDA GRAS approval and will be used in proper proportions in increasing amounts to improve the flavor of foods.

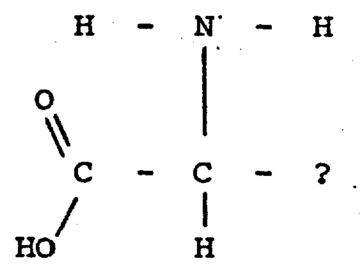
Protein Hydrolysates and Flavor Enhancers

Data Sheets

Page

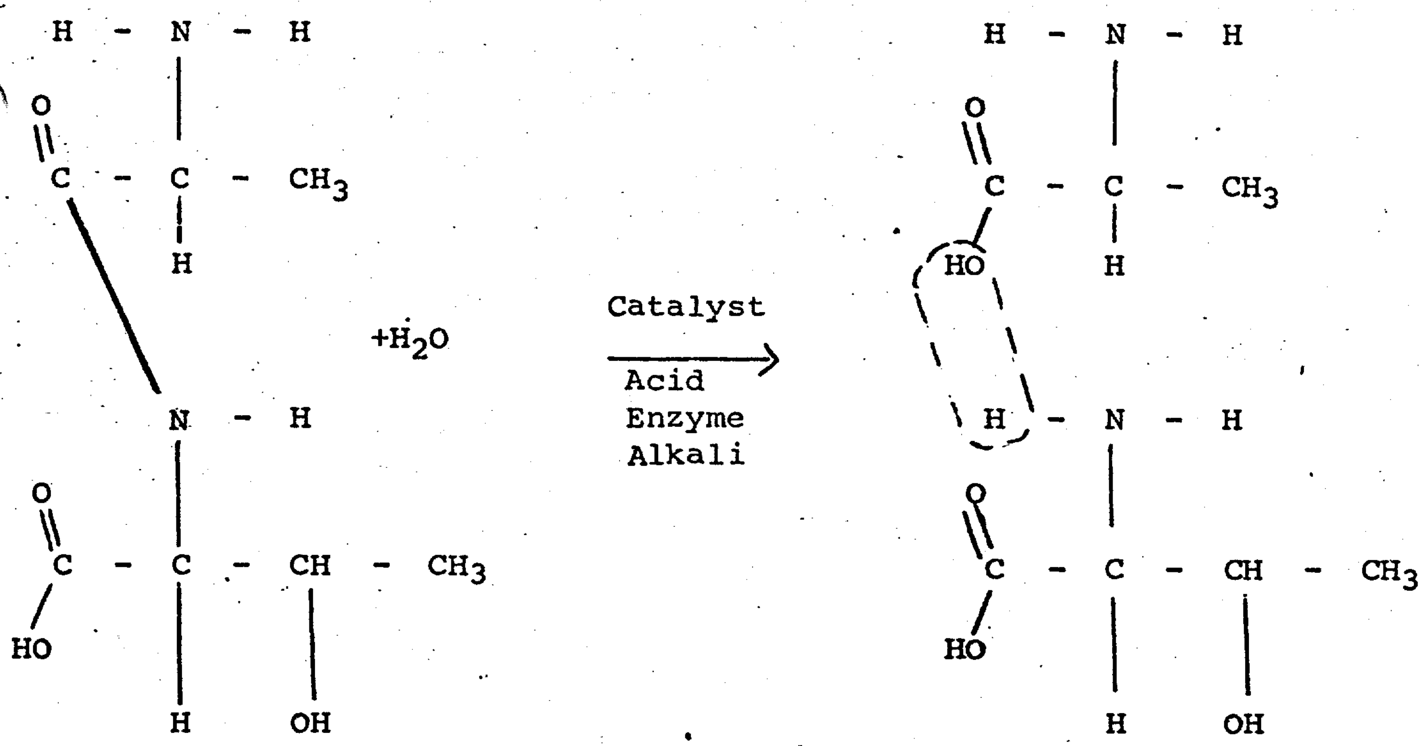
Protein Hydrolysates - Chemical Structure

Chemical Structure Common to Amino Acids
(An acid -COOH group and an amino - NH_2 group on an adjoining carbon atom)



Proteins are combinations of amino acids held together by a peptide linkage.

Hydrolysis of Proteins

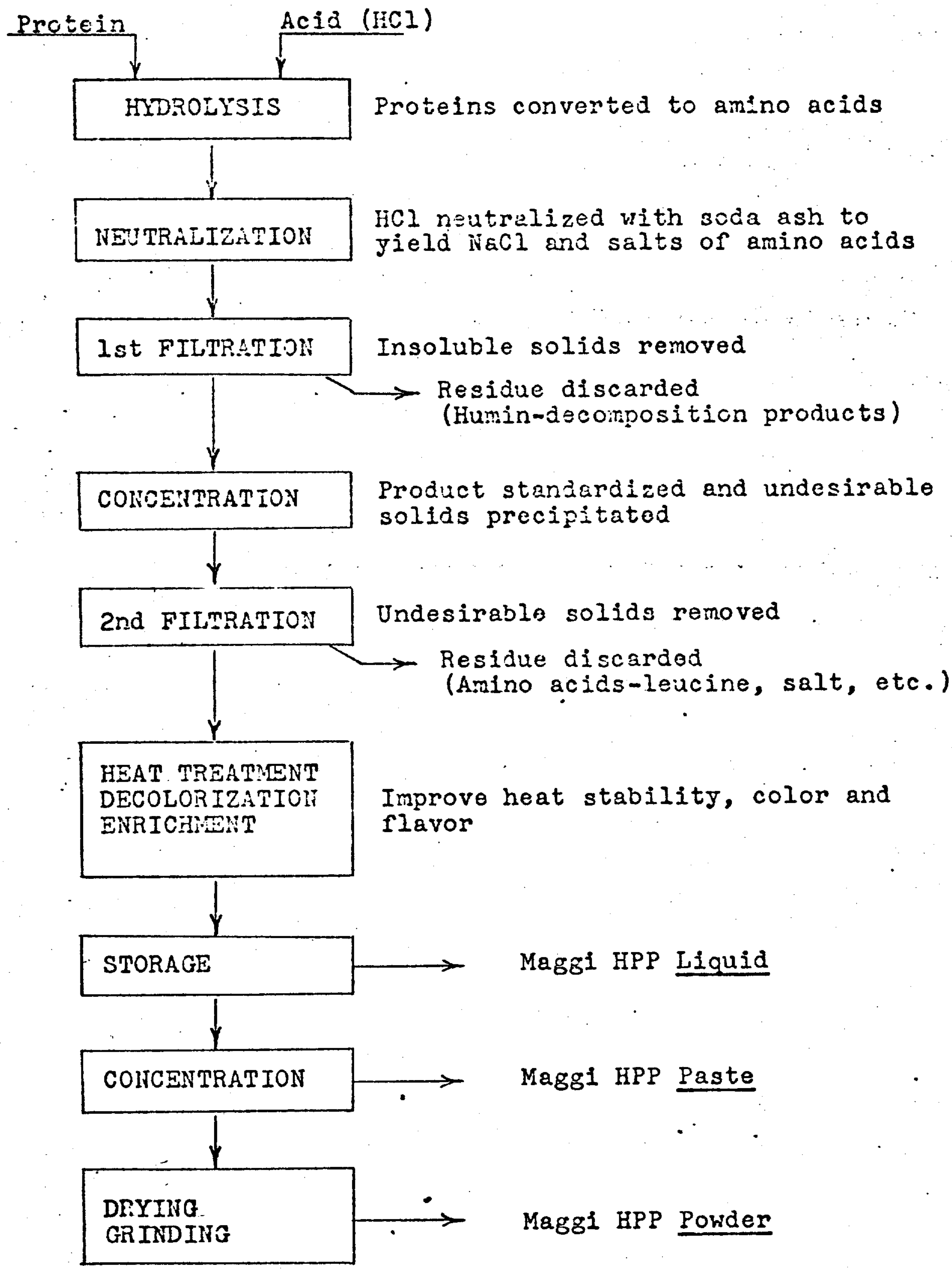


Peptide Linkage
Protein

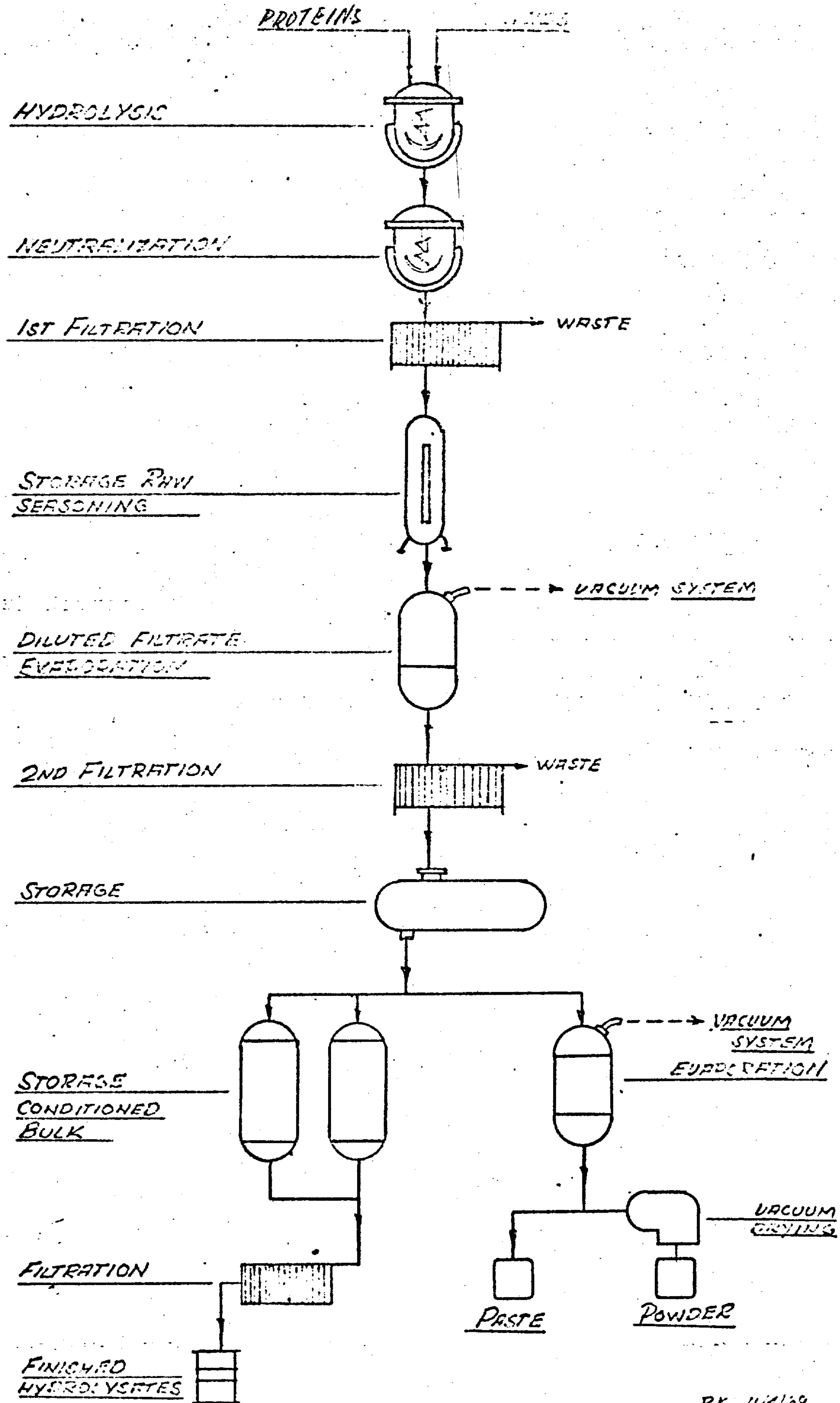
Amino Acids

MAGGI BULK HYDROLYZED PLANT PROTEIN

PROCESSING STAGES



FLOW DIAGRAM - HYDROLYZED PLANT PROTEIN



HYDROLYZED PLANT PROTEIN

Typical Analytical Composition

	Liquid	Paste	Powder
Total Solids %	40.0	85.0	98.0
Ash %	20.0	42.5	49.0
Organic Solids %	20.0	42.5	49.0
Chloride, calc.as NaCl %	17.5	37.0	42.5
Total Nitrogen %	2.80	6.00	6.90
Protein (Nx6.25) %	17.5	37.5	43.0
MSG %	5.0	10.6	12.0
pH	5.3	5.2	5.1

HYDROLYZED PLANT PROTEIN

AMINO ACID COMPOSITION

	Brand X %	Brand Y %
Valine	1.7408	1.7485
Lysine	1.5624	0.8697
Threonine	1.4277	1.5002
Leucine	1.6003	2.0192
Isoleucine	1.0201	0.8037
Phenylalanine	1.2781	1.7268
Histidine	0.7007	0.8283
Arginine	1.4206	1.4030
Aspartic Acid	3.0017	2.9841
Serine	1.8953	2.1891
Glutamic Acid	20.2145	11.4599
Proline	3.0457	4.4480
Glycine	1.2542	1.1566
Alanine	3.2414	4.0773
Tyrosine	0.2965	0.2670
Total Amino Acid	43.700	37.421
Total Protein (Nx6.25)	43.8	37.9

YEAST EXTRACT

Typical Analytical Composition

	Standard Series (Dark & Light) Paste	Standard Series (Dark & Light) Powder	Special Series (Dark & Light) Paste	Special Series (Dark & Light) Powder	Low Sodium Series Paste	Low Sodium Series Powder
Total Solids %	80.0	97.0	80.0	97.0	80.0	97.0
Ash %	23.0	28.0	22.0	26.5	10.0	12.0
Organic Solids %	57.0	69.0	58.0	70.5	70.0	85.0
Chloride, calc. as NaCl %	15.0	18.0	13.0	15.7	2.0	3.4
Total Nitrogen %	7.80	9.45	7.50	9.10	9.35	11.0
Protein (Nx6.25)%	48.7	59.5	46.9	57.0	58.5	69.0
MSG %	4.5	5.45	21.0	25.5	4.3	5.2
pH	5.5	5.5	5.5	5.5	5.6	5.6

PROTEIN HYDROLYSATES

Hydrolyzed Plant Proteins - Blends of naturally occurring amino acids and salts of amino acids.

Protein Content (Nx6.25) - May vary from 30 to 45%.

Sodium Content - May vary from 12 to 18%.

pH of aqueous soln. - 5.0 to 5.4.

Solubility in water at 20° C. - 40% by wt.

Forms - Liquid, Paste or Powder
The powder is hygroscopic and must be stored in moisture protective containers at temperature under 95° F. Products are available ranging in color from light to dark.

Stability - High quality grades are resistant to decomposition during heat processing. There is no flavor degradation when held for long periods under ambient conditions in a moisture protective pack.

Occurrence - Hydrolyzed Plant Proteins (amino acids) are the building blocks of proteins and are therefore found in all natural proteins.

Use Levels - 0.2% to 1.5%, in most foods about 0.5%.
Hydrolyzed plant proteins have a synergistic effect when used in combination with nucleotides.

Flavor - Have meat-like flavor covering a wide spectrum. Excellent for imparting pleasing flavor profiles. Can be used to intensify, blend or suppress other flavors. Ideally suited for use in enhancing and potentiating food flavor. Have a nucleotide sparing effect.

MONOSODIUM GLUTAMATE

Chemical Structure

$$\left.\begin{array}{ccccc} O & = & C & - & OH \\ & & | & & \\ H & - & C & - & NH_2 \\ & & | & & \\ H & - & C & - & H \\ & & | & & \\ H & - & C & - & H \\ & & | & & \\ O & = & C & - & ONa \end{array}\right\} .H_2O \quad (C_5H_8NO_4Na.H_2O)$$

Molecular Weight - 187

Purity - 99% minimum

Sodium Content - 12.3%

Solubility in water at 25° C. - 37% by wt.
at 60° C. - 50% by wt.

pH of aqueous soln. - 7.0

Form - Free flowing, white crystals, non-hygroscopic.

Stability - Do not decompose in heat processing. In high acid foods, MSG may be converted to glutamic acid and lose its flavor enhancing properties.

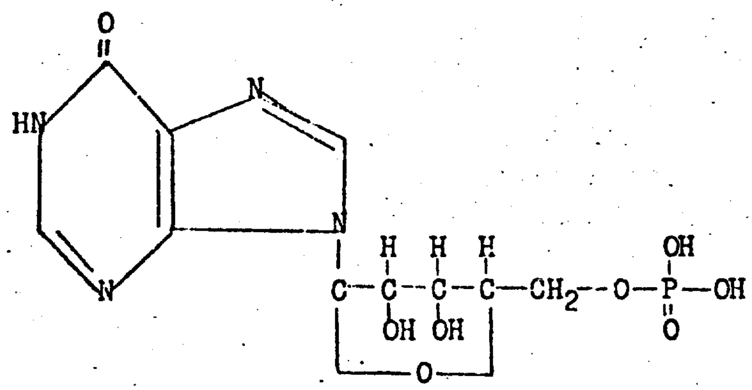
Occurrence - Small amounts in virtually all foods; also combined in all natural proteins.

Use Levels - 0.05% to 0.5%, in most foods about 0.2%.
(Use just enough to enhance the natural flavor)

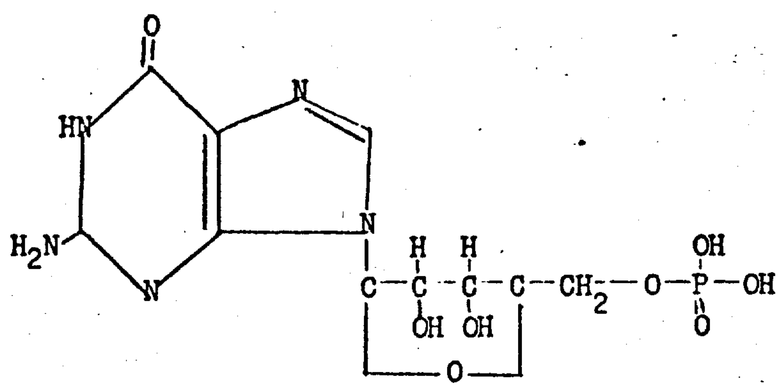
Flavor - Has a sweet, saline taste accompanied by some astringency. Can be used to enhance inherent flavors in a wide variety of foods without adding a flavor of its own.

NUCLEOTIDES 5' IMP and 5' GMP

Figure 1: Chemical Structures of 5'-inosine monophosphate and 5'-guanosine monophosphate.



5'-Inosine monophosphate



5'-Guanosine monophosphate

NUCLEOTIDES

Disodium Inositate and Disodium Guanylate
(5' IMP) (5' GMP)

Grades Available - 5' IMP, 5' GMP, or blends of 50% 5' IMP and 5' GMP

Purity - 98% minimum

Solubility in water at 20° C. - 20% by Wt.
at 60° C. - 45% by Wt.

pH of aqueous soln. - 7.0 - 8.5

Form - White crystalline powder, slightly hygroscopic.

Stability - Nucleotides when used in a pH range of 4 to 7 are not decomposed by heating for 1 hr. at 212° F. or by strong oxidizing or reducing agents with heating for 30 minutes. Under retorting conditions of 240° F. for 1 hr., there is about a 10% loss due to decomposition. Nucleotides may be decomposed by phosphatase enzyme systems in certain kinds of food depending upon processing conditions.

Occurrence - Nucleotides are the building blocks which form nucleic acids and are therefore found naturally in many foods.

Use Levels - 0.002% to 0.02% on an as served basis. Nucleotides have a synergistic effect when used in combination with HPP or MSG.

Flavor - Have a sweet, meaty flavor note. Are most effectively used in combination with either HPP or MSG to utilize a synergistic effect. Can be used to give a bodying effect and to suppress some flavors.

THE NESTLÉ COMPANY

INC.

FOOD PRODUCT DEVELOPMENT - NUTRITION P-6213

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42.

PROTEIN HYDROLYSATES AND FLAVOR ENHANCERS

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PROTEIN HYDROLYSATES AND FLAVOR ENHANCERS

Suggested examination question:

Hydrolyzed Plant Protein, Monosodium Glutamate and Nucleotides are food ingredients widely used to enhance and intensify flavor. Select one of these ingredients and discuss such aspects as: historical background, manufacture, composition, flavor characteristics and utilization.

Observations on Protein Digestion in vivo

V. FREE AMINO ACIDS IN BLOOD PLASMA OF RATS FORCE-FED ZEIN, CASEIN, OR THEIR RESPECTIVE HYDROLYZATES[1]

CARL PERAINO[2] AND ALFRED E. HARPER[3]
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT Concentrations of free amino acids in the portal and systemic blood plasma of rats force-fed zein, casein, or their respective hydrolyzates were determined in an effort to show a relationship between the change in plasma amino acid pattern and the relative digestibility of the proteins. Force-feeding zein had little effect on the concentrations of free amino acids in the portal and systemic plasma but force-feeding casein caused a substantial increase in the concentrations of free amino acids in the portal plasma. When hydrolyzates of these proteins were force-fed, much larger increases in the plasma free amino acid concentrations were observed with the largest responses occurring after ingestion of casein hydrolyzate.

In an earlier part of this study (1) the rates of disappearance of different proteins from the gastrointestinal tracts of rats fed a single meal were compared. Casein and zein emptied from the stomach at slightly different rates but much more nitrogen accumulated in the small intestines of rats fed zein than could be accounted for by the difference between the rates at which the 2 proteins emptied from the stomach. Further investigation (2) revealed that the residual nitrogen recovered after feeding zein was mainly in an insoluble fraction and probably consisted of undigested protein.

The objective of the present study was to determine whether differences in the digestibility of these proteins would be reflected in the concentrations of free amino acids in the blood plasma. Comparisons were made between the concentrations of free amino acids in the plasma of rats force-fed a given amount of protein (zein or casein) and of others force-fed the same amount of nitrogen from the corresponding protein hydrolyzates.

METHODS

The proteins were commercial products. Hydrolyzates were prepared by autoclaving (15 pounds pressure) 100 g of protein in 2 liters of 4 N sulfuric acid for 24 hours; neutralizing the resulting solution with solid calcium hydroxide; filtering off the precipitate of calcium sulfate; decolorizing the filtrate with Norite;[4] and removing water by evaporation.

Male white rats (200 g) which had previously been fed a stock diet and had been starved for 24 hours were force-fed an amount of test protein or hydrolyzate which contained 0.16 g of nitrogen (i.e., an amount equivalent to 1 g of pure protein). The hydrolyzates were not supplemented with amino acids. The test nitrogen sources were administered as aqueous suspensions or solutions (10% w/v). Blood samples were taken from the portal vein and heart of individual rats at successive time intervals after feeding. The procedure has been described (3). Equal quantities of heparinized portal blood taken from 2 rats at each interval were combined; systemic blood samples (obtained by heart puncture) were similarly combined and the cells were removed from each pooled sample by centrifugation.

Free amino acids in the resulting plasma samples were then determined quantitatively by a paper chromatographic

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80: 270-278
Jul. 1963

procedure (4). Results are presented for the following amino acids: glutamic acid, glutamine, serine, threonine, proline, alanine, glycine, valine, leucine + isoleucine (not separated on the chromatogram), and lysine.

RESULTS

The quantity of the particular amino acid in the protein or hydrolyzate that the rats received is shown in the legend for each figure. The concentrations of the designated amino acids in both the portal and systemic plasma at each half-hour interval after force-feeding the test protein or its corresponding hydrolyzate are indicated by the points on the curves. The points on the vertical axes of the figures directly above the letter F indicate the concentration of the particular amino acid in both the portal and systemic plasma of animals kept without food for 24 hours (fasting concentration).

Zein and zein hydrolyzate. Force-feeding zein did not cause the concentration of glutamic acid in either the portal or systemic plasma to increase much above the fasting value, but after administration of the hydrolyzate a substantial increase was observed in the portal plasma and a smaller increase in the systemic plasma (fig. 1).

The concentration of alanine in both portal and systemic plasma (fig. 1) increased above the fasting value after force-feeding zein, but larger increases occurred in both after force-feeding the hydrolyzate, with the portal concentration showing the greater response. Although the amount of hydrolyzate given contained less alanine

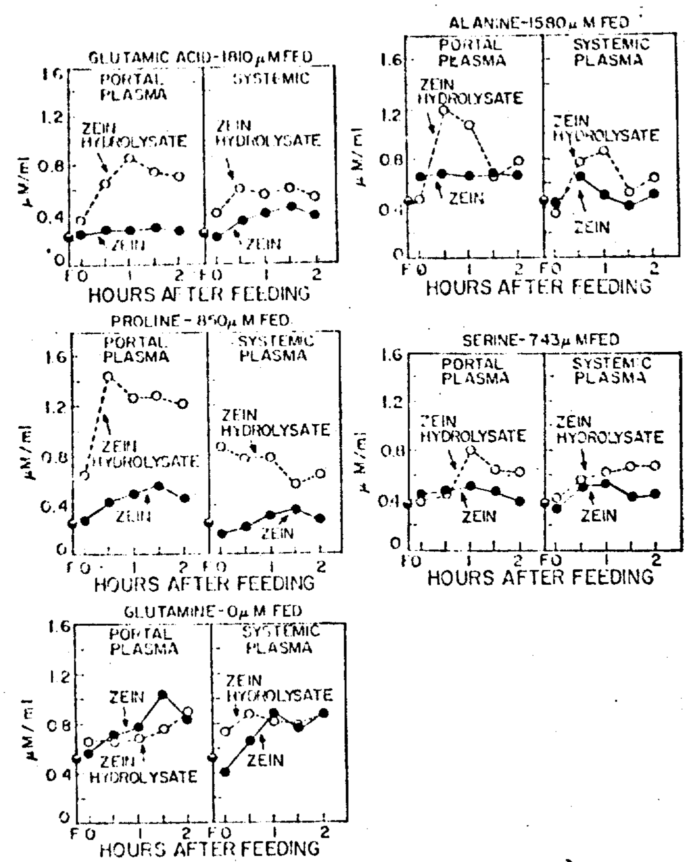

Fig. 1. Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding zein or zein hydrolyzate.

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(1580 μmoles) than glutamic acid (1810 μmoles), alanine concentration rose to a maximum of 1.2 μmoles/ml, whereas glutamic acid concentration rose to only 0.85 μmoles/ml.

The concentration of proline (fig. 1) increased only slightly in the portal and systemic plasma after force-feeding zein, but increased much more in both after force-feeding the hydrolyzate. Proline increased less in the systemic than in the portal plasma. The amount of hydrolyzate administered contained only 850 μmoles of proline compared with 1580 μmoles of alanine and 1810 μmoles of glutamic acid, but the concentration of proline in the portal plasma increased more than the concentrations of either alanine or glutamic acid.

The effect of prior hydrolysis of zein on plasma serine concentration (fig. 1) was much less than its effect on the plasma concentrations of glutamic acid, alanine and proline. Also, differences between the plasma serine concentrations of groups fed zein and zein hydrolyzate did not occur until one hour after feeding.

The concentration of glutamine (fig. 1) gradually increased in both portal and systemic plasma of rats force-fed zein or zein hydrolyzate, although this amino acid is not present in zein. The plasma concentration of glycine (fig. 2), another amino acid which is not found in zein, did not change appreciably from the fasting value in any of the trials.

When zein was force-fed the concentration of valine did not increase above the

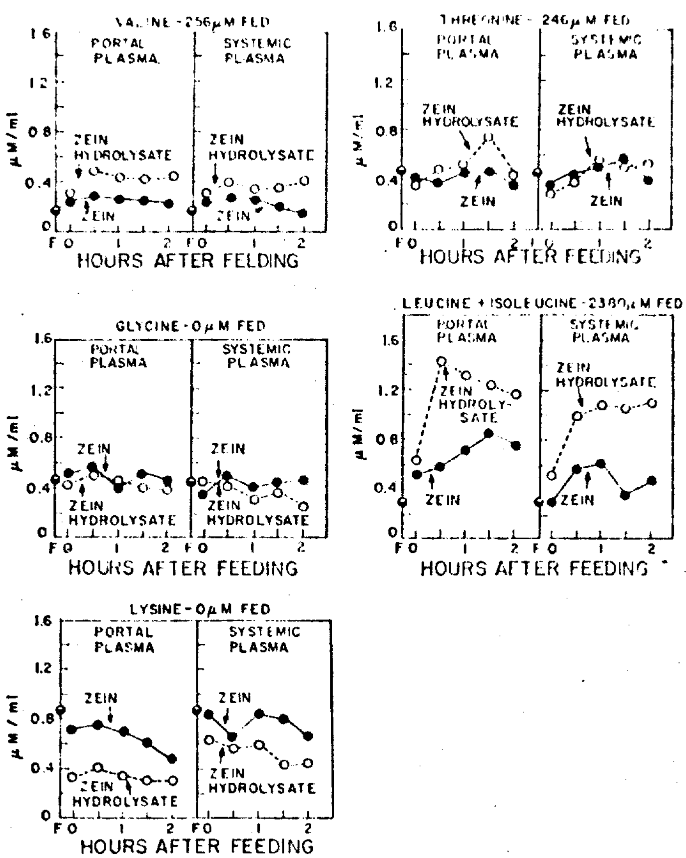

Fig. 2 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding zein or zein hydrolyzate.

fasting value in either the portal or systemic plasma (fig. 2). A small increase in valine concentration occurred in both portal and systemic plasma of the rats force-fed the zein hydrolyzate. Similar results were obtained for threonine (fig. 2). Both valine and threonine are present in relatively low concentrations in zein (both 3%).

The concentration of leucine plus isoleucine in the plasma after feeding zein or zein hydrolyzate is shown in figure 2. The value for the amount fed represents the sum of the concentrations of the 2 amino acids in the meal. The concentration of leucine plus isoleucine increased in both the portal and systemic plasma after force-feeding zein. However, the increase was much greater in both after administration of zein hydrolyzate. The curve for leucine plus isoleucine in the portal plasma after feeding zein hydrolyzate appears to be similar to the corresponding curve for proline (fig. 1) although the hydrolyzate contained almost 3 times as much leucine plus isoleucine (2380 μmoles) as proline (850 μmoles). The systemic plasma curves for these amino acids differ markedly, with the leucine plus isoleucine values being much higher than those for proline after the first hour.

The effect of force-feeding zein or zein hydrolyzate on the plasma concentration of lysine, an indispensable amino acid not present in zein, is also shown in figure 2. In contrast with the results shown for the dispensable amino acids, glutamine and glycine, the plasma concentration of lysine decreased after the administration of zein or zein hydrolyzate, the decrease being greater with the latter.

Casein and casein hydrolyzate. Glutamic acid is the amino acid present in highest concentration (23%) in casein. Although a relatively large quantity (1568 μmoles) of this amino acid was force-fed in the casein or casein hydrolyzate, its concentration did not increase appreciably in either case (fig. 3). Also, plasma glutamic acid concentration increased to the same extent whether the meal contained casein or casein hydrolyzate and the concentration of glutamic acid in the portal plasma increased only slightly above that in the systemic plasma. Comparison of the portal plasma glutamic acid concentrations of rats that received intact zein, or casein (figs. 1 and 3) shows that the value for those receiving casein increased more.

Although the casein meal contained less alanine (453 μmoles alanine ingested) than the zein (1580 μmoles alanine ingested) the concentration of alanine in both portal and systemic plasma increased more after force-feeding casein (fig. 3) than zein (fig. 1). When intact casein was given, the concentration of alanine in the portal plasma increased as much as when the hydrolyzed protein was fed, but the maximal concentration was not reached quite as early. The alanine concentration in the systemic plasma did not increase after force-feeding casein hydrolyzate.

The concentration of proline in the portal plasma increased substantially after force-feeding casein and a somewhat smaller increase occurred in the systemic plasma (fig. 3). Comparable increases were not observed when intact zein (fig. 1) was given. A greater increase occurred in the concentration of proline in the portal plasma after force-feeding the casein hydrolyzate (fig. 3). This increase was comparable to that observed after force-feeding zein hydrolyzate, although the amount of proline ingested in the casein hydrolyzate was somewhat greater (1071 μmoles for casein hydrolyzate as opposed to 850 μmoles for zein hydrolyzate). The increase in the concentration of proline in the systemic plasma after the ingestion of hydrolyzates of zein or casein was always much less than the increase in the proline concentration in the portal plasma.

The concentration of serine increased to the same extent in both portal and systemic plasma when casein was force-fed (fig. 3). This increase was somewhat greater than that observed when zein was force-fed (fig. 1), although in the latter case more serine was ingested (743 μmoles as opposed to 656 μmoles ingested when casein was fed). When casein hydrolyzate was given, the increase in plasma serine was similar to, but slightly less than, that observed after giving intact casein.

Force-feeding casein caused a marked increase in the concentration of glutamine

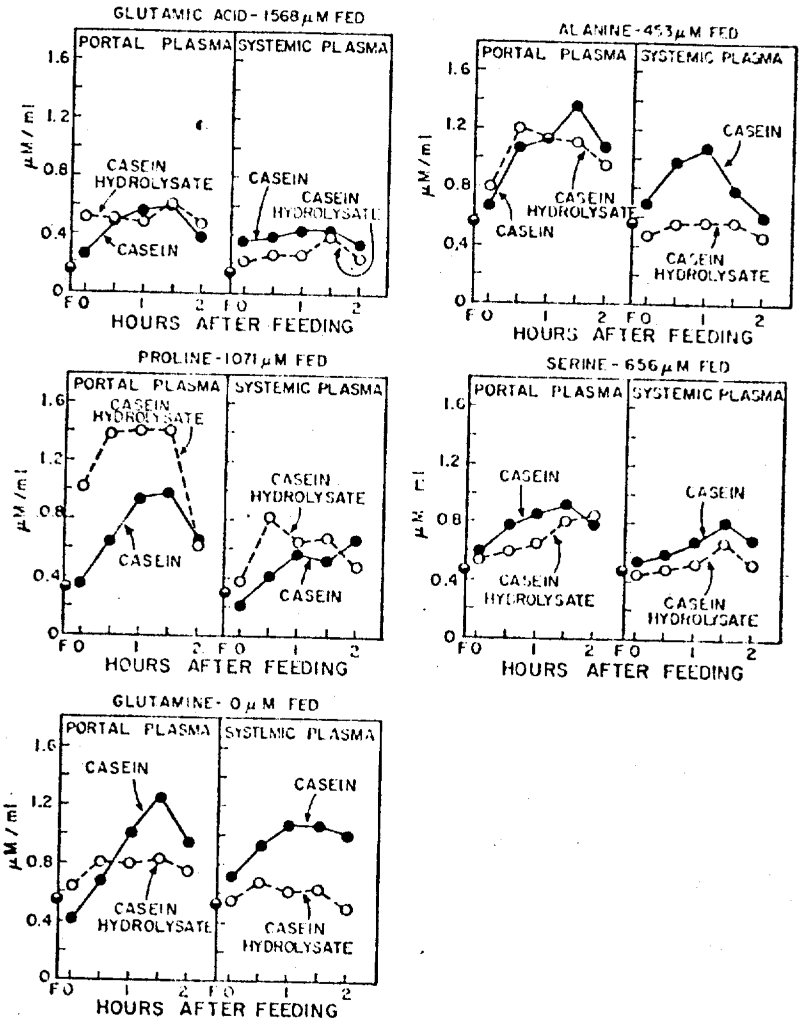

Fig. 3 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding casein or casein hydrolyzate.

in both portal and systemic plasma; yet force-feeding the hydrolyzate had much less effect (fig. 3).

Force-feeding casein resulted in an increase in the concentration of valine in both portal and systemic plasma, the increase in the systemic plasma being somewhat smaller (fig. 4). When casein hydrolyzate was force-fed, much larger increases in valine concentration occurred in both portal and systemic plasma, the concentration again being lower in the systemic plasma.

The concentration of threonine in the portal plasma increased well above the fasting value when casein was force-fed (fig. 4), but the systemic plasma concentration did not increase appreciably. The portal plasma concentration increased more rapidly during the first hour after force-feeding the hydrolyzate, but the maximum was not quite as high as that observed when intact casein was given.

The concentration of glycine did not increase appreciably in either portal or systemic plasma after force-feeding casein

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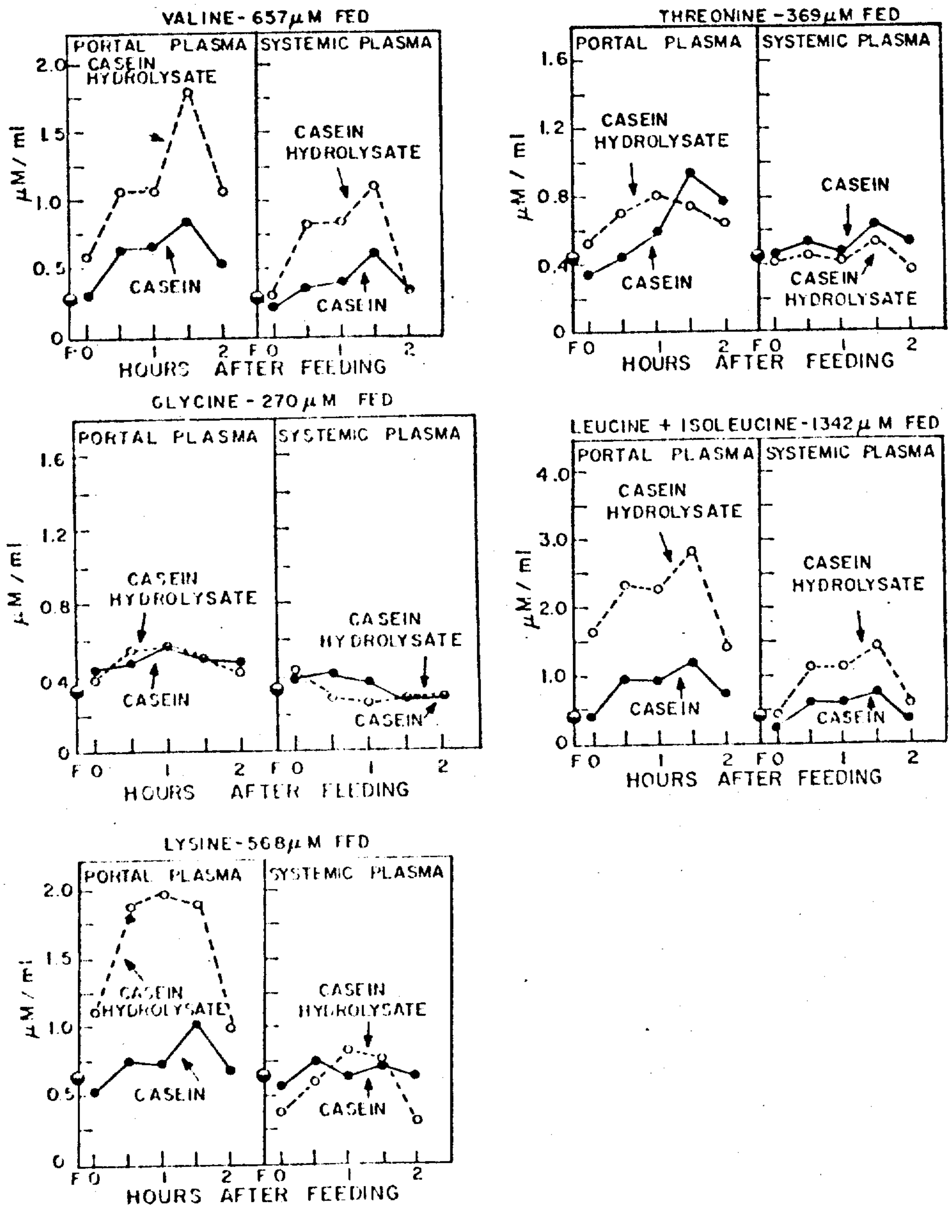

Fig. 4 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding casein or casein hydrolyzate.

or casein hydrolyzate (fig. 4). The curves closely resemble those for glycine after the feeding of zein which contains no glycine (fig. 2). Casein contains only 2% of glycine.

The concentration of leucine plus isoleucine increased moderately in the portal plasma and only slightly in the systemic plasma after casein was force-fed (fig. 4). The concentration of leucine plus isoleucine increased much more in the portal plasma after administration of casein hydrolyzate; however, the general shape of the curve closely resembled that obtained with intact casein. The concentration of leucine plus isoleucine in the systemic plasma after force-feeding the hydrolyzate was greater than that observed after intact casein but was still well below the maximal leucine plus isoleucine concentration observed in portal plasma. When casein hydrolyzate was force-fed, the concentration of leucine plus isoleucine in the plasma increased more in proportion to the amount ingested (1342 μmoles) than when zein hydrolyzate (containing 2380 μmoles) was fed (fig. 2).

The concentration of lysine (fig. 4) increased moderately in the portal plasma, but changed very little from the fasting value in the systemic plasma after casein was force-fed. Force-feeding casein hydrolyzate caused a rapid increase in the concentration of lysine in the portal plasma, but the concentration in the systemic plasma was not appreciably affected.

DISCUSSION

The changes which occur in the concentrations of free amino acids in the plasma after the ingestion of dietary protein depend upon the amino acid composition of the dietary protein; the rate at which the protein empties from the stomach; the rate of release of free amino acids from the protein during digestion; the rates of absorption of the amino acids; the extent to which the amino acids are metabolized by the intestinal tissues during absorption; and the rates of removal of the absorbed amino acids from the blood. Guggenheim et al. (5, 6) have suggested that, although the concentration of amino acids in the portal blood may serve as a guide to amino acid availability, the relationship between portal blood concentrations and availability is neither direct nor simple. Denton and Elvehjem (7) and Morrison et al. (8) have shown that differences in the availability of amino acids in dietary protein may be reflected in blood amino acid concentrations.

An overall comparison of the amino acid patterns in the portal plasma after the test proteins or their hydrolyzates were fed shows that the amino acids of zein and about one-half of those of casein were absorbed into the portal blood more rapidly when hydrolyzates were fed. This indicates that digestion of the protein was for the most part a more limiting factor than amino acid absorption in determining the rate of entry of the dietary amino acids into the portal blood in these experiments.

The relatively low concentrations of amino acids in the portal plasma after feeding intact zein indicates that the amino acids of zein were less available for absorption than those of casein. This might be expected owing to the high concentration of nitrogen, apparently from undigested protein, found in the intestinal contents of animals fed zein (12), and is in agreement with the observation of Goldberg and Guggenheim (6).

In these experiments even when the diet contained hydrolyzed protein some amino acids were not absorbed into the portal blood in direct proportion to their concentrations in the test meal. In this connection Orten et al.[5] and Pinsky and Geiger (9) found that the rate of absorption of individual amino acids by the intestine was affected by the presence of other amino acids, and Orten[6] found that the overall rate of absorption of an amino acid mixture, as well as the pattern of the mixture that is absorbed, are affected by the qualitative composition of the mixture present in the intestine.

The frequent lack of correlation between the rates of absorption of dietary amino acids (as manifested by increases in their concentrations in the portal plasma) and the quantities of these amino acids ingested may thus be due in part to the effects of the particular amino acid mixture present in the intestine on the absorption process. The presence of relatively large quantities of certain amino acids may depress the absorption of other amino acids. For example, when zein hydrolyzate is fed, the proportion of leucine ingested is very large. The presence of a relatively large concentration of leucine in the intestinal contents could conceivably depress the rates of absorption of other amino acids.

Also, the rates of absorption of individual amino acids differ markedly (10–12). These differences could have considerable

[5] Orten, A. H., K. Korzumi and D. J. France 1951 Federation Proc., 10: 390 (abstract).
[6] Orten, A. H. 1961 Federation Proc., 20: 2d.

effect on the response of the plasma amino acid pattern to the feeding of protein or protein hydrolyzate.

The observation that the ingestion of a large quantity of casein or its hydroyzate caused only a relatively small increase in the concentration of glutamic acid in the portal plasma is in agreement with results obtained by Dent and Schilling (12) with dogs fed casein. In other experiments the administration of glutamic acid alone resulted in a relatively small increase in the concentration of this amino acid in the portal plasma (3). As was previously suggested (3) the relatively small increase in portal plasma glutamic acid may be the result of a relatively low rate of absorption of glutamic acid from the intestinal lumen (11) coupled with a high rate of metabolism of this amino acid by the intestinal cells during absorption (13).

The decrease in the concentration of plasma lysine observed after feeding zein, and the still larger decrease observed after feeding the hydrolyzate, suggests that the free lysine in the plasma was drawn into the cells during stimulation of protein synthesis caused by the influx of amino acids from the intestine. The more pronounced lowering effect of the hydrolyzate would then be expected as a result of more rapid absorption of the free amino acids. Wu (14) also observed a decrease in the concentration of lysine in the plasma of rats fed a zein diet for 12 days; and Hill et al. (15) in experiments on chicks found that the addition of 15% of zein to a diet containing 9.5% of soybean protein resulted in a marked decrease in the concentration of lysine in the plasma.

The lack of change in the concentration of glycine after the feeding of zein or zein hydrolyzate indicates either that the influx of the dietary amino acids had no effect on the metabolism of glycine; or that the utilization of glycine by the tissue increased but *de novo* synthesis of glycine compensated for the increased utilization so that no net change in the concentration of glycine in the plasma was observed.

The increase in plasma glutamine concentration after the feeding of zein or zein hydrolyzate indicates synthesis of this amino acid in response to the production of ammonia during the metabolism of the ingested amino acids (16). However, the larger increase in plasma glutamine observed after feeding casein as opposed to its hydrolyzate cannot be adequately explained on this basis.

When considered as a whole, the results of this investigation indicate that the response of the plasma amino acid pattern to the ingestion of a protein or its hydrolyzate is complex, and depends on many factors in addition to the digestibility of the dietary nitrogen source. However, comparison of the concentrations of free amino acids in the portal plasma after feeding the unhydrolyzed proteins does show that the amino acids in casein are more available for absorption than those of zein and indicates that such comparisons may provide useful information about the relative digestibility of dietary proteins.

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ACCUMULATION OF GLUTAMIC ACID IN THE ARCUATE NUCLEUS OF THE HYPOTHALAMUS OF THE INFANT MOUSE FOLLOWING SUBCUTANEOUS ADMINISTRATION OF MONOSODIUM GLUTAMATE[1]

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Abstract—Monosodium L-glutamate was injected subcutaneously into 4-day-old mice at a dose of 2 mg/g body wt. The infants were killed at sequential intervals after injection, the brains were frozen, and samples of the arcuate nucleus (NA), ventromedial hypothalamus (VMH) and lateral thalamus (LT) were micro-dissected from lyophilized sections for glutamate assay. Blood glutamate levels were also determined for comparison with brain levels of glutamate at corresponding post-injection intervals. Glutamate levels in the NA steadily increased to reach a peak value of 110·9 mmol/kg dry wt. at 3 h following injection, whereas the highest levels reached in the VMH or LT were about 41·7 mmol/kg dry wt. Return to control values of about 25 mmol/kg dry wt. occurred gradually over a period of 12–15 h in all three brain regions. Blood glutamate concentrations peaked rapidly, reaching a maximum of 40 mM within 15 min but returned precipitously to near-baseline values (below 1 mM) in the 1–3 h interval after injection. We discuss possible mechanisms to account for the transient marked accumulation of subcutaneously administered glutamate in the NA and how this might relate to the selective destruction of arcuate neurons which occurs simultaneously.

MONOSODIUM glutamate, administered orally or subcutaneously, at a dose of 0·5–4·0 mg/g body wt. in mice and rats destroys neurons in the inner layers of the retina (LUCAS and NEWHOUSE, 1957; POTTS, MODRELL and KINGSBURY, 1960; COHEN, 1967; OLNEY, 1969*a*) and arcuate nucleus (NA) of the hypothalamus (OLNEY, 1969*b*, 1971; OLNEY and HO, 1970; BURDE, SCHAINKER and KAYES, 1971; EVERLY, 1971). OLNEY (1969*b*) reported that mice treated during infancy with monosodium glutamate exhibited obesity and neuroendocrine abnormalities as adults. Certain other acidic amino acids also known to have neuroexcitatory properties (namely, aspartic, cysteic, cysteine sulphinic and homocysteic acids) produce lesions identical to those produced by glutamate (OLNEY and HO, 1970; OLNEY, HO and RHEE, 1972).

The lesions in the retina and NA are similar (OLNEY, 1969*a*, 1971) being characterized by rapid swelling of neuronal dendrites and cell bodies followed by nuclear pyknosis, all occurring within 6–8 h after administration. Cellular edema subsides by 12 h and many of the necrotic neurons are already phagocytized by 24 h. By the fourth post-treatment day phagocytosis and biological degradation of degeneration

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[2] Recipient of Research Career Development Award MH-38894.
Abbreviations used: LT, lateral nucleus of the thalamus; NA, arcuate nucleus; VMH, ventromedial nucleus of the hypothalamus.

products are virtually complete and by day 8, there are no striking histological abnormalities within the area other than the marked decrease in number of neurons. Glial and ependymal cells within the arcuate region show distinct swelling as early as 15 min after treatment but this is a reversible event.

The purpose of the present study was to ascertain whether the NA of the infant mouse has a tendency to accumulate subcutaneously administered glutamate during periods of lesion formation. Our suspicion that this might be so was corroborated by a 4-fold increase in glutamate levels in the NA at 3 h, with only a slight elevation in the immediately adjacent ventromedial nucleus of the hypothalamus (VMH) or in a more remote region, the lateral nucleus of the thalamus (LT).

EXPERIMENTAL

Animals. Eighteen litters of 124 male and female Webster Swiss albino mice (4-days old at the start of the experiments) were used. According to the split-litter technique, 76 mice were injected subcutaneously with a 10% (w/v) aqueous solution of L-glutamic acid, monosodium salt (Sigma Chemical Co., St. Loius, Mo.) at a dose of 2 mg/g body wt. Four control mice were untreated. At 0 (controls), 7·5, 15, and 30 min, and 1, 3, 6, 9, 12 and 18 h after injection the mice were decapitated and the heads were immediately frozen on solid carbon dioxide and stored at −90°C. There were 4 mice in each of the groups, except for the 6-h group in which there were 12 mice and for the 9-, 12- and 18-h groups, each with 8 mice. For measuring blood glutamate the remaining 48 mice were treated as already described and decapitated at 0 (controls) 15 and 30 min, and 1, 3 and 6 h after injection at which times blood was collected in heparinized capillary tubes.

Preparation of tissues and chemical assays. In a cold room at −15°C the frozen heads were blocked by removing unwanted portions with a scalpel blade. To avoid traumatizing the arcuate region the base of the skull was left intact. The resulting block consisted of the entire thalamus and hypothalamus plus portions of the directly subjacent skull. These blocks were mounted on aluminium tissue holders with brain paste and transverse sections 40 μm thick were cut at −17°C and freeze-dried, as described by LOWRY (1953). Sections were stored evacuated at −30°C until assayed. Blood samples were centrifuged at 25°C and the plasma was frozen and stored at −30°C.

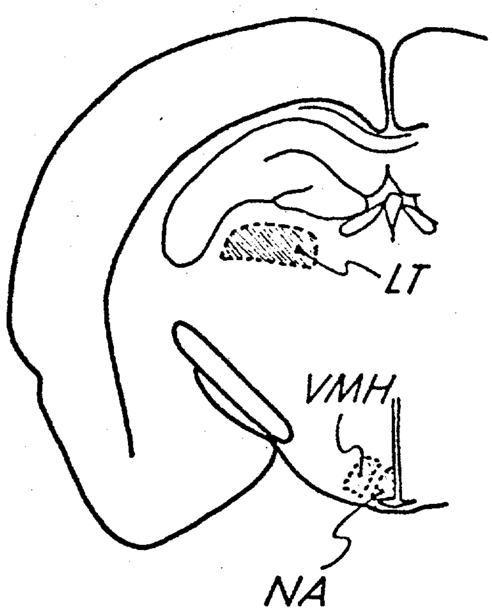

FIG. 1.—Sketch of a coronal section of mouse brain showing the approximate locations of the arcuate nucleus (NA), ventromedial hypothalamus (VMH) and lateral thalamus (LT) from which our samples were micro-dissected and weighed (LOWRY, 1953) and assayed for glutamic acid (YOUNG and LOWRY, 1966).

FIG. 2.—Coronal sections of brain from 4-day-old mice taken 3 h after subcutaneous injection with physiological saline (left) or with monosodium L-glutamate (right), 2 mg/g body wt. The experimental section illustrates neuronal degeneration in the NA produced by glutamate. No abnormalities were evident in the immediately adjacent VMH. Brains were fixed, sectioned and stained according to histological procedures described by OLNEY (1971). Magnification ×100.

Bilateral samples weighing 0·2–1·2 μg were dissected by hand from the NA, VMH and LT (Fig. 1) of frozen-dried sections from each mouse and weighed on a quartz-fibre microbalance, as described by LOWRY (1953). Glutamic acid was assayed in all tissue samples and in the 0-, 3- and 6-h blood samples by the histochemical method described by YOUNG and LOWRY (1966), except that during cycling we used only one-tenth of the amount of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glutamate dehydrogenase (EC 1.4.1.3) recommended by LOWRY and PASSONNEAU (1963) for maximal cycling, and cycling was carried out for only 30 min. Glutamate in blood samples taken 15 and 30 min and 1 h after injections was measured with the 'macro' method of YOUNG and LOWRY (1966).

RESULTS

Because of the marked edema that developed in the NA of the infant mice in the 30-min to 6-h groups we could distinguish this region even in unstained sections as a zone appearing different from the remainder of the brain. However, to accentuate the effect for purposes of illustration, we also prepared stained sections from perfused animals (Fig. 2). It was a striking feature of the hypothalamic lesion induced by a 2 mg/g dose of glutamate that only the cells in the NA were affected, with those in the immediately adjacent VMH left entirely unaltered.

For purposes of comparison with blood levels, the levels of glutamate in control brain samples were converted from mmoles/kg dry wt. to mmoles/kg wet wt. by multiplying the dry wt. values by 0·12, since the water content of the infant rodent brain (rat) is approximately 88% (PICCOLI, GRYNBAUM and LAJTHA, 1971). In control animals the glutamate content was virtually identical in the NA (3·35 mmol/kg wet wt.), VMH (3·27 mmol/kg) and LT (3·28 mmol/kg) with little variation among animals. The corresponding levels expressed per kg dry wt. were 27·9, 27·2 and 27·4 mmol, respectively (Fig. 3).

In the NA of experimental animals the glutamate levels doubled within 15 min, reached a maximal mean level of 110·9 mmol/kg dry wt. at 3 h, decreased to near

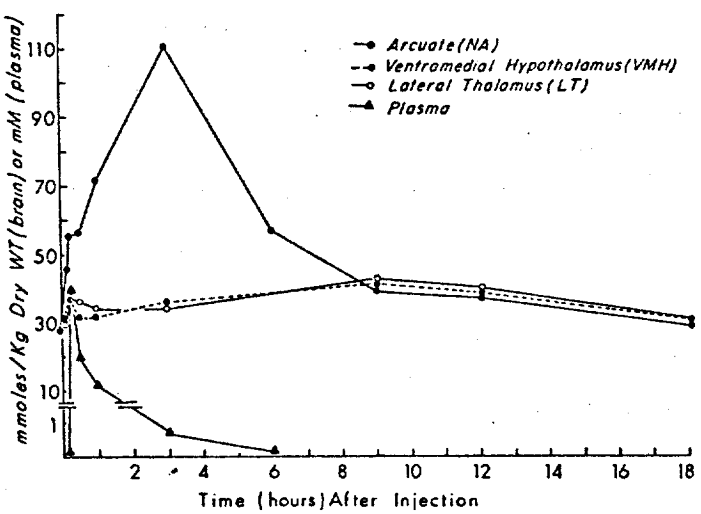

FIG. 3.—Blood concentrations and regional distributions of glutamic acid in brains of 4-day-old mice at various times after the subcutaneous injection of monosodium L-glutamate, 2 mg/g body wt. Each point represents the mean of glutamic acid (mmoles/kg dry wt. or mM) for blood from 8 mice or for bilateral samples of NA, VMH and LT from 4 mice at 0 (controls) through 3 h, 12 mice at 6 h and 8 mice in each group thereafter. Glutamic acid was measured as described by YOUNG and LOWRY (1966) with the modifications outlined in the text section on Methods.

control levels (39·2 mmol/kg dry wt.) by 9 h, but did not reach control levels of approximately 27 mmol/kg dry wt. until 12–18 h (Fig. 3). Although the mean level of glutamate in the NA at 6 h was 56·7 mmol/kg dry wt., it was at this time that the greatest variation among animals occurred, with values ranging from 29·2 to 165·8 mmol/kg dry wt. For this reason, tissues from 12 mice were assayed at 6 h and from 8 mice at each time thereafter. Glutamate concentrations in the NA of experimental animals are expressed only in terms of dry wt. (as they were actually measured) because the dry/wet wt. conversion ratio available for normal brain would not give an accurate reflection of the true wet wt. concentrations of glutamate in the edematous NA of experimental animals.

In contrast to the marked increase in the levels of glutamate in the NA, there was only an increase of less than 16·7 mmol/kg dry wt. (2 mmol/kg wet wt.) above control levels in the VMH and LT. In these regions glutamate levels peaked rapidly to about 37·5 mmol/kg dry wt. at 15 min, decreased slightly until 3 h, and then increased to a maximal value of about 41·7 mmol/kg dry wt. by 9 h after injection. A circadian, periodic fluctuation in blood concentrations of glutamate was described in mice by FEIGIN, DANGERFIELD and BEISEL (1969). Therefore, we propose that the gradual secondary upswing in the VMH and LT curves may reflect a circadian periodicity for brain levels of glutamate, so that in both the NA and these other regions, the levels of glutamate at 9 h could possibly be considered normal.

The concentration of glutamate in blood rose sharply from control values of 0·12 mM to a peak mean concentration of 40·1 mM by 15 min after injection. A rapid decline, however, brought blood glutamate levels to the normal range (less than 1 mM) in the 1–3 h interval, during which time glutamate levels in the NA continued to increase steadily.

DISCUSSION

The marked accumulation of glutamate in the NA and the minimal accumulation in the adjacent VMH, coupled with histopathological evidence that neurons of the NA but not those of the VMH swell massively and die over a 3–6 h period following the injection of L-glutamate, is consistent with the view that glutamate ions play a relatively direct role in producing this cytotoxic syndrome. It is puzzling, however, that the arcuate region should progressively concentrate glutamate, even against a steep tissue-to-blood concentration gradient, while its neurons are being destroyed by the process. In the 15- to 30-min interval following glutamate administration, when blood levels probably transiently exceeded those of the NA, it is possible that the influx of glutamate occurred by passive transfer across permeable blood–brain barriers. For several hours thereafter, however, the level of glutamate continued to rise in the NA, despite blood concentrations estimated to be only one-tenth to one-twentieth those in the NA. This phenomenon suggests that an active transport mechanism was responsible for at least part of the uptake of glutamate by the NA.

It is a characteristic of brain tissue *in vitro* that it actively concentrates glutamate, a phenomenon which is presumed to be intracellular and is associated with swelling of the brain slice and with an influx of water and K^+ (STERN *et al.*, 1949; TERNER *et al.*, 1950; PAPPIUS and ELLIOTT, 1956; TOWER, 1958; TAKAGAKI, HIRANO and NAGATA, 1959; BOURKE and TOWER, 1966). Electrophysiological experiments *in vitro* suggest that exposure of brain slices to glutamate induces depolarization and increased permeability of neural membranes to Na^+ (BRADFORD and MCILWAIN, 1966; HARVEY and MCILWAIN, 1968). Assuming that there is an ineffective or absent blood–brain barrier to glutamate in the arcuate region of infant mouse brain, this tiny portion of the brain would be directly exposed to the glutamate concentration in the blood, much as a brain slice *in vitro* is exposed to the glutamate in the incubation medium. In either the *in vivo* or *in vitro* situation it might be a toxic effect of the glutamate ion itself which causes the brain tissue to concentrate the ion. Alternatively, the phenomenon could reflect some physiologic mechanism for concentrating glutamate intracellularly, in which case we would need to postulate absence of a shut-off mechanism to protect the cell against incorporation of toxic excesses.

From microelectrophoretic studies *in vivo* it is known that the introduction of glutamate into the extracellular surroundings of neurons stimulates neuronal firing (CURTIS, 1969). The neurotoxic syndrome under consideration here may represent an exaggeration of this neuroexcitatory phenomenon. Instead of reversibly depolarizing the neural membrane by a transient slight increase in extracellular glutamate, as occurs in the electrophoretic situation (CURTIS, PHILLIS and WATKINS, 1960), the subcutaneous administration of glutamate to infant mice, by producing a sustained elevation in the extracellular concentration of glutamate in the arcuate region, may cause a persistent depolarization and prolonged increase in permeability of neural membranes. Such a set of events probably could lead to necrosis of involved neurons as a result of the persisting loss of protection normally afforded by the usual permeability characteristics of the plasma membrane of the neuron.

Support for the thesis that the neuroexcitatory and neurotoxic phenomena associated with administration of glutamate *in vivo* are linked by a similar underlying mechanism is provided by recent studies on molecular specificity (OLNEY *et al.*, 1972). In these experiments it was shown that a select group of acidic amino acids (aspartic, cysteine sulphinic, cysteic and homocysteic acids and certain substituted synthetic congeners of these compounds) which share with glutamic acid both a similarity in molecular structure and an ability to excite neural membranes also share with glutamic acid an ability to selectively necrose arcuate neurons when given subcutaneously to infant mice. It has been postulated that one of the functions of glutamic acid in the brain is that of a neurotransmitter (KRNJEVIČ and PHILLIS, 1963; CURTIS, 1969). If this were the case, it would be essential that the glutamate released at nerve endings be either metabolically inactivated extracellularly or immediately returned to the intracellular compartment to prevent prolonged stimulation of the post-synaptic apparatus. The need for immediate re-uptake, i.e. for maintaining the extracellular environment free of glutamate ion, may underly the rapid uptake of glutamate by tissue slices *in vitro* or by cells of the NA *in vivo*.

There have been a number of efforts to clarify whether any exchange of glutamate between blood and brain occurs. Our data pertaining to glutamate uptake by the VMH and LT are consistent with other studies on infant rodents (THURSTON and WARREN, 1971; HIMWICH, PETERSON and ALLEN, 1957) in which a small elevation of whole brain levels of glutamate was detected following parenterally administered L-glutamate. Others, carrying out similar experiments on adult rodents consistently found that no net increase in the brain content of glutamate was measurable following parenteral loading (SCHWERIN, BESSMAN and WAELSCH, 1950; LAJTHA, BERL and WAELSCH, 1959) but interpretations of the phenomenon differ. Initially, it was assumed that blood–brain barriers against glutamate are poorly developed at birth, but become so well established thereafter that glutamate is simply excluded from entering the adult brain. The more recent studies by LAJTHA *et al.* (1959) of the uptake of radioactive glutamate suggest rather that glutamate enters adult brain from blood but is not detected as a net increase in brain glutamate because an equal amount is immediately either returned to the blood or converted metabolically to other compounds. Regardless of which interpretation is correct, in the adult and even to a large extent in the infant, most of the brain is apparently protected by a mechanism geared to maintaining a steady-state with regard to brain levels of glutamate. Our data suggest that the NA of the infant mouse lies outside this 'protection'. Measurement of enzyme activities concerned with glutamate metabolism in the NA of infant brain for comparison with activities in other more 'sheltered' regions should help to clarify whether differing metabolic capabilities underly the greater tendency of the NA to accumulate glutamate despite the dire cytotoxic consequences.

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EXPERIMENTS IN THE TOXICITY OF AND TOLERANCE FOR MONOSODIUM GLUTAMATE WITH A TEST OF AVOIDANCE RESPONSE CONDITIONING

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The use of glutamic acid or of monosodium glutamate (MSG) has been proposed several times in cases of mental retardation, but there is a notable divergence of opinions both in regard to the efficacy of such therapy and in regard to the dosage. Various authors in fact had used doses that ranged from 5 to 140 g daily (21,1).

The MSG is also used in food as an additive "emphasizing the flavors" or as an actual food ingredient. It has been calculated that the total daily intake through all possible reasonable uses of the product (except for therapeutic uses) is on the order of 0.7 g, that is 0.01 g/kg in the case of the adult (3). This value is notably less than the limits established by the World Health Organization: in fact, the competent Committee has concluded that the product "could be administered in the amount of 0-120 mg/kg (unconditional ADI), applicable to the whole population except for children under 1 year of age" (20).

Indications of undesired secondary effects have not been lacking either following the administration of MSG for therapeutic purposes (10), or following the intake of food which contains it as an additive or ingredient (the so-called Chinese restaurant syndrome)(17).

In the evaluation of the therapeutic and toxicological data, it is necessary to remember that the administration of MSG leads to a rapid increase of the hematic concentration of glutamic acid, followed within about 2 hours ~~later~~ by a return to almost normal values(9). On the other hand, plasmatic values of glutamic acid 50 times higher than the normal one can be accompanied by concentrations in the brain that are not significantly different from those for the control(11). In spite of this, results have been reported in favor of the stimulating effect of MSG on the central nervous system, for example an increase in spontaneous motor activity (7) and in the capacity for learning of the rat in a labyrinth (22). On the other hand, more recent results so far reported only in summary form, seem to deny that MSG can exert influence on the behavior of rats and monkeys (16).

The experiments described here have been conducted to evaluate the effects of MSG on the conditioned avoidance response behavior in rats. The acute effects have been studied on the adaptations of previously trained animals, comparing them with the effects caused by equivalent doses of sodium chloride (NaCl), either the effects of the treatment in the training period or finally the tolerance phenomena that develop with repeated treatment.

It is noted that following the prevailingly negative results of preliminary experiments, the tests described here have been conducted with ~~high~~ large doses of the products. These experiments are presented not only because they show the scarcity of the effects of MSG but also because they form a further example of experimental procedures for the study of possible toxic effects of various types of products on the conditioned behavior of the laboratory animal.

The Experimental Part

Before the beginning of the experiments on conditioned behavior, tests for acute toxicity were made with MSG and NaCl orally on rats and mice according to the conventional method.

For the conditioning tests there were used male white rats raised in our Institute, belonging to a strain derived from the Wistar strain. The animals weighed 200-250 g at the beginning of the experiments.

Apparatus

A series of pieces of apparatus ~~were~~ was used for the study of bidirectional avoidance response conditioning, with automatic programming and registration (shuttle-box)(4). Each group of apparatus includes a rectangular box, 490x270x225 mm, connected to a system for programming and to a register of results. The box, without any inside ~~separation~~, has a floor formed by metal bars with a diameter of 3 mm through which there can be administered an electric shock (unconditioned stimulus, US), which in these experiments has always been 1.5 mA. The floor is balanced on a central axis in order to permit microswitches to indicate the presence of the animal in the left or right part of the cage. In the center of the ceiling, there is a light source with 10 w, that furnishes the conditioned stimulus (CS).

The Conditioning Program

The animals have been subjected daily, except Sunday, to conditioning sessions of 25 minutes in each one of which they received 50 tests at regular intervals of 30 seconds. Each test began with the lighting of the CS, followed after 5 seconds later by the US. A crossing response during the first 5 seconds of the presentation of the CS (a conditioned response, CR) put an end to the same CS and prevented the administration of the US; a crossing response after the beginning of the US put an end simultaneously to the CS and US. The crossing of the cage during an interval between tests was punished with an electric shock so that the animal was compelled to return to that part of the cage in which the preceding test was concluded.

Experimental Procedure

a) The effects of MSG and NaCl on previously trained animals.- For this experiment there were used exclusively rats that in a prolonged training period had reached high ~~un~~stable levels of adaptation (CR > 80% for three consecutive sessions). All of these animals had received orally, before each training session, a volume of water equal to that of the solution of MSG or of NaCl subsequently administered. For the actual pharmacological experiment, 4 groups of animals were treated orally 30 minutes before the test, with MSG 5 g/kg, MSG 10 g/kg, NaCl 1.7 g/kg (a dose equivalent to that of 5 g/kg of MSG) and NaCl 3.4 g/kg (a dose equivalent to 10 g/kg of MSG) respectively.

b) The effects of MSG on the acquiring of conditioning - This experiment was conducted in two parts: in the first, comparison was made of a group treated daily, orally, 30 minutes before each session with 5 g/kg of MSG and a control group treated with water; in the ~~latter group~~ second part a group treated with 10 g/kg of MSG and another control group were subjected to training tests. Each group was composed of 18 animals without previous experience; they were subjected to tests for 8 days, with one day's interval between the first 5 sessions and the last 3 sessions. Furthermore, in the second part of the experiment (that is the one with the dose of 10 g/kg), the animals were subjected to tests for another 3 days in order to study the effects of the so-called change of state (14), that is to say, of the administration of MSG to the group previously treated with water and vice versa.

c) The tolerance for MSG. - The checking, in the course of the preceding experiment, of phenomena of tolerance to MSG required the performance of another experiment designed to give preliminary indication on the response mechanisms of tolerance itself. In fact, previous experiments have shown that the disappearance of the effect of a psycho-drug on behavior following repeated treatments can be due ~~either~~ to a true tolerance insofar as it can be verified in animals treated after~~wards~~ rather than before each test (6,5). Therefore, a group of 12 rats previously trained was treated for 12 days with water before the tests and with MSG after the tests (5 g/kg daily for the first 6 days and 10 g/kg daily for the other 6 days). Subsequently, 10 g/kg of the product were administered 30 minutes before the begining of the test for 2 days. Another group of 14 rats was treated instead only with water before and after the tests for 12 days and then with MSG before the tests for the last 2 days of the experiment.

Results

Toxicity

The LD50 of MSG and of NaCl are indicated in Table I.

The acute effects of MSG and NaCl on previously trained animals

Table II indicates the frequency of the conditioned avoidance responses before and after the treatments. It appears evident that only very ~~high~~ large doses of MSG and of NaCl, i.e., higher than one half of the respective LD50 caused a significant reduction in the frequency of the CR.

Table 1

Acute toxicity of monosodium glutamate (MSG) and of sodium chloride (NaCl) when administered orally.(*)

	Topino Mouse	Ratto Rat
MSG	19,20 (22,84-16,13)	16,60 (18,90-14,50)
NaCl	4,00 (4,38-3,65)	4,20 (4,43-3,98)

(*) LD50 (g/kg) and the respective limits of confidence up to 95% calculated according to the method of Litchfield and Wilcoxon (12).

Table II

The effects of monosodium glutamate (MSG) and of sodium chloride (NaCl) on the avoidance response behavior in previously trained rats.

		N.	Average of the CR SE Media delle RC (in %) ± E.S.			
			Controls Controlli	Trattati	Difference Differenza	p (*)
MSG	5 g/kg	21	77,4 ± 3,2	71,7 ± 4,6	5,7 ± 3,0	n.s.
	10 g/kg	18	86,1 ± 3,4	51,2 ± 6,9	34,9 ± 5,2	<0,001
NaCl	1,7 g/kg	16	90,0 ± 2,0	82,6 ± 4,2	7,4 ± 4,2	n.s.
	3,4 g/kg	17	88,9 ± 1,5	66,1 ± 6,7	22,8 ± 6,5	<0,005

*Levels of significance (student's t through paired observations, with two tails).

<u>The effects of MSG on the acquiring of conditioning.</u>

The results of the first part of this experiment are not presented in detail inasmuch as differences worthy of note were not discovered between the control group and the group treated with 5 g/kg of MSG. For example, the average percentage of CR along the whole curve of the eight days of tests was equal to 40 CR in the control group and to 37 in the group treated with MSG.

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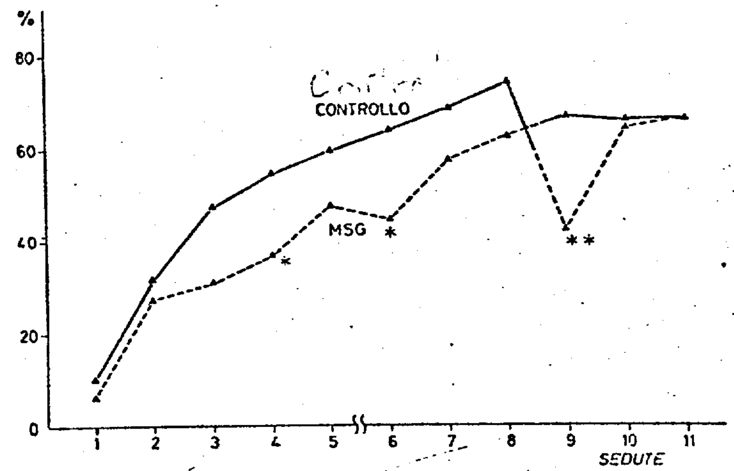

Fig. n. 1

Fig. 1

The effects of monosodium glutamate (MSG 10 g/kg p.o.) on the acquisition of avoidance response conditioning in the rat.

In the ordinate is indicated the percentage of avoidance responses. The asterisks for the fourth and sixth sessions indicate statistically significant differences between the controls and the treated animals. (Student's t with two tails for independent samples, $p < 0.05$ and > 0.02). After the eighth session the treatment was suspended; furthermore, the same dose of MSG was administered to the rats previously used as controls before each one of the last three sessions. The double asterisk for the ninth session indicates a significant decrease in the frequency of conditioned responses in the previously untreated group (a comparison between the ninth and eighth sessions, t through paired observations, $p < 0.005$).

In the second part of the experiment, conducted with a very much ~~higher~~ larger dose of MSG (i.e., administering every day more than one-half of the LD50), a retardation was observed in the acquisition of avoidance response behavior, this retardation however being very slight and statistically significant only in the fourth and sixth sessions(Fig. 1).

The results obtained in these sessions following the acquisition session confirmed what was already observed in the previous experiments, i.e., the significant reduction of frequency of the CR following the administration of 10 g/kg of MSG to previously trained animals. However, the repetition of this treatment quickly led to a disappearance of the effect, demonstrating the rapid establishment of a tolerance for the product. The suspension of administration of MSG in the other group did not cause any variations worthy of note in the frequency of CR.

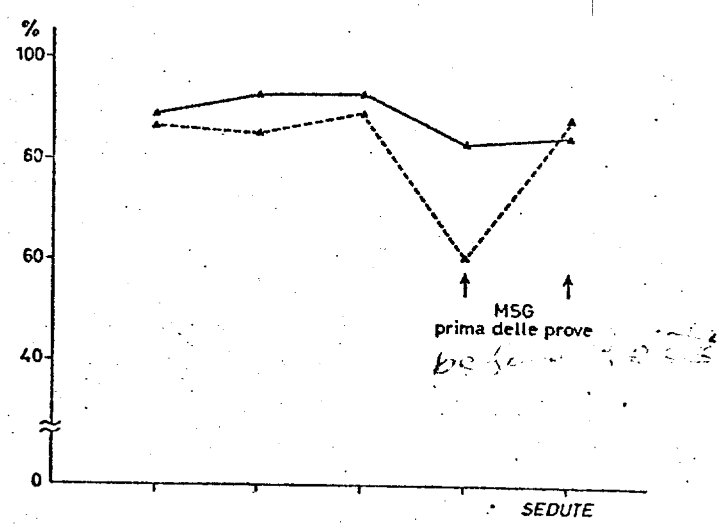

Fig. n. 2

Tolleranza al glutammato monosodico (MSG)

Fig. 2

Tolerance for monosodium glutamate (MSG).

MSG (10 g/kg p.o.) was administered in the last two days of the experiment 30 minutes before the tests to two groups of rats: the first group treated only with water (the broken line) during the preceding twelve days, the second group previously treated with MSG (continuous line) after each test (5 g/kg for six days and 10 g/kg for another six days). There is noted in the second group a notable ~~weakening~~ attenuation of the decrease in frequency of the conditioned responses normally caused by the administration of 10 g/kg of MSG before the test. The data concern the percentage of conditioned responses in the last five days of the experiment.

Tolerance for MSG

Fig. 2 shows clearly that the effects of a large ~~high~~ dose of MSG, administered 30 minutes before the test (see the control group) are weakened not only following the repeated administration of the product before the test but also following the repeated administration of the product after the test. In fact, even if, in both groups, there was a significant decrease in the frequency of CR in the first session preceded by treatment with MSG, nevertheless this decrease was much less marked in the group which had previously received the product several times after the test. The difference between the decreases observed in the two groups yielded a statistically significant result ($p < 0.02$ with the Mann-Whitney non-parametric test, used because of the presence of a significant heterogeneity of the variants).

Discussion

The results of the experiments reported above clearly indicate that the effects of the MSG on the conditioned avoidance response behavior are of slight importance until doses with toxic effects are administered, i.e., ~~higher~~ greater than one half of the LD50. This conclusion is valid not only with respect to experiment with previously trained animals, but also with respect to the experiments in which the product was administered during the learning period in order to increase the probability of demonstrating a possible depressive effect.

It is well known that a decrease in adaptations after a treatment with psycho-drugs can be due not to an actual depressive effect but to a so-called change of state, where, in a given situation, the correct performance of the exercise is state-dependent (14). In this case, a decrease in the adaptation is observed either when the product is administered to animals not treated previously, or when the administration is suspended in a previously treated group. This occurrence seems excluded in the case of MSG: in fact, no decrease was observed in the frequency of CR after the suspension of the administration of the product to those animals which ~~who~~ had ~~previously~~ ~~acquired conditioned avoidance response behavior in continuous treat-~~ acquired avoidance response behavior under conditions of continuous treatment.

It is also noted that in the course of learning, no stimulating effect by the product was observed when it was administered in sub-toxic ~~eff~~ doses (5 g/kg), in contrast to what has been previously reported with respect to learning in a labyrinth (22). But it is necessary to be more cautious in excluding the presence of a facilitating effect that often appears only in tests or under conditions that are very special, more cautious i.e., than in excluding ~~for not to exclude~~ the presence of a depressive effect with ~~respect to the~~ regard to which various tests yield relatively less heterogeneous results.

It is furthermore noted that already at the second administration, the same greater dose of MSG (10g/kg) loses a large part of its effect. It is well known that phenomena of this kind may be due to various causes, i.e., either to a tolerance in the classic sense of the word --- as in the case of narcotics and of LSD-25 --- or to the coming into action of compensation systems that correct the behavior deficit without ~~which (or before) a true tolerance has developed.~~ a true tolerance having developed or before it develops. This last occurrence has been demonstrated several times in the course of repeated treatment with amphetamine (18) or scopolamine (5,8,2). A distinction between the two types of desensitivization is generally made by means of suitable experiments, i.e.: a) experiments that demonstrate (or fail to demonstrate) notable differences in the speed of desensitivization from one test to another; b) experiments that demonstrate whether or not there are notable differences in the speed of desensitivization according to whether the repeated treatments are given before each test session or at another time, in general after the end of each session. In the case of MSG, it has appeared obvious that the repeated treatment after each test ~~notably~~ perceptively decreased the effect of the product when ~~it was begun~~ the administration of the product was begun before the test. Therefore it ought to be concluded that the evidence is in favor of the ~~rapi~~ rapid development of a true tolerance.

This phenomenon tends on the other hand to diminish the significance of the essentially negative results obtained in the experiments in which the product was administered from the very beginning of the learning process (an absence of effect with 5 g/kg, a not very marked depressive effect with 10 g/kg). In fact, on the first day, the adaptations are always very low even in the control group, which leaves little room for the demonstration of a retardation in the learning process. On ~~the~~ following days the lack of depressive effects or their scarcity could be attributed to the rapid establishment of a tolerance.

Finally it is necessary to remember that, because of the scarcity of data in the literature as well, such essentially negative results do not lend themselves at the present time to extrapolations and to generalizations for other situations and species. In particular, as it was recalled in the beginning, the ~~notably perceptible~~ elevation of hematic concentrations of glutamic acid even when of notable amount is not accompanied in adult animals by an increase in the concentration of amino acids in the brain (11). Therefore the negative evidence obtained through behavior tests in the adult animal is not in itself in opposition to ~~with that from~~ the evidence of damage caused to the central nervous system by MSG if administered during the period of development (13,19,15). Obviously, it remains to be determined if these last effects are to be attributed to a greater sensitivity of the central nervous system in the development ~~phases~~ and organization phases or simply to a more effective penetration of the product through the hemato-encephalic barrier.

ESPERIENZE DI TOSSICITÀ E DI TOLLERANZA AL MONOSODIOGLUTAMMATO CON UN SAGGIO DI CONDIZIONAMENTO DI SALVAGUARDIA

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RIASSUNTO. — *Sono stati studiati gli effetti del glutammato monosodico (MSG) sul comportamento bidirezionale di salvaguardia nella shuttle-box, con un segnale luminoso come stimolo condizionato (SC), uno shock elettrico di 1,5 mA come segnale incondizionato (SI), un intervallo SC-SI di 5 secondi, un intervallo fra singole prove di 30 secondi ed un intervallo fra sedute successive (di 50 prove ciascuna) di circa 24 ore.*

Solo una dose assai elevata di MSG (10 g/kg p.o.) *si è dimostrata capace di ridurre la frequenza delle risposte di salvaguardia, sia in animali precedentemente addestrati, sia durante il periodo dell'apprendimento. Inoltre si è osservata una rapida comparsa della tolleranza nel corso di trattamenti ripetuti. Alcune delle esperienze sono state eseguite con procedimenti atti a mettere in evidenza eventuali effetti legati al cosiddetto cambiamento di stato (o dipendenza dallo stato:* change of state, state-dependency) *ed a distinguere fra la tolleranza farmacologica vera e propria e la desensibilizzazione dovuta a compenso per altra via dei deficit di comportamento. Queste prove hanno escluso un ruolo significativo della dipendenza dallo stato nel caso del MSG, ed hanno mostrato che l'attenuazione degli effetti del prodotto è dovuta ad una vera e propria tolleranza.*

SUMMARY. — *A shuttle-box task with a light CS, a 1.5 mA shock as US, a 5-second CS-US interval, a 30-second inter-trial interval, and daily sessions of 50 trials each was used to study the effects of monosodium glutamate (MSG) on avoidance behaviour in rats. Only a very high dose of MSG* (10 g/kg p.o.) *was effective in depressing avoidance acquisition and performance. Furthermore, tolerance appeared rapidly with repeated treatment. Additional experiments were carried out designed to investigate the role of state-dependency, and to discriminate between true pharmacologic tolerance and desensitization due to other types of compensation of drug-induced deficits. These tests excluded a significant role of state-dependency in the case of MSG, and showed that the attenuation of MSG effects is due to true pharmacologic tolerance.*

L'impiego dell'acido glutammico o del glutammato monosodico (MSG) è stato più volte proposto nei casi di ritardo mentale, tuttavia vi è una notevole divergenza di opinioni sia riguardo all'efficacia di tale terapia, sia riguardo al dosaggio. Vari AA., infatti, hanno usato dosi che andavano dai 5 ai 140 g al giorno (21, 1).

Il MSG viene anche adoperato in campo alimentare come additivo « esaltante i sapori » o come componente alimentare vero e proprio. È stato calcolato che l'assunzione totale quotidiana attraverso tutti i ragionevoli usi possibili del prodotto (esclusi quelli terapeutici) è dell'ordine di 0,7 g, cioè 0,01 g/kg nel caso dell'adulto (3). Questo valore è notevolmente inferiore ai limiti stabiliti dall'Organizzazione Mondiale della Sanità: infatti il Comitato competente ha concluso che il prodotto « potesse essere somministrato nella misura di 0-120 mg/kg (*unconditional ADI*, assunzione quotidiana ammissibile senza condizioni), applicabile a tutta la popolazione esclusi i bambini al di sotto di un anno di età » (20).

Non sono mancate segnalazioni di effetti secondari indesiderati sia a seguito della somministrazione di MSG a scopo terapeutico (10), sia a seguito dell'assunzione di cibi che lo contengono quale additivo od ingrediente (cosiddetta sindrome del ristorante cinese) (17).

Nel valutare i dati terapeutici e quelli tossicologici occorre ricordare che la somministrazione di MSG conduce ad un rapido aumento della concentrazione ematica dell'acido glutammico, seguita entro circa due ore dal ritorno a valori pressochè normali (9). D'altra parte, valori plasmatici di acido glutammico 50 volte superiori a quelli normali possono accompagnarsi a tassi cerebrali non significativamente diversi da quelli di controllo (11). Ciò malgrado, sono stati riportati risultati a favore di un'azione stimolante del MSG sul sistema nervoso centrale, ad esempio l'aumento dell'attività motoria spontanea (7) e della capacità di apprendimento del ratto nel labirinto (22). Viceversa risultati più recenti, sinora riportati soltanto in forma di riassunto, sembrano negare che il MSG possa influire sul comportamento del ratto e della scimmia (16).

Le esperienze qui descritte sono state condotte per valutare gli effetti del MSG sul comportamento condizionato di salvaguardia nel ratto. Sono stati studiati sia gli effetti acuti sulle prestazioni di animali precedentemente addestrati, confrontandoli con quelli provocati da dosi equivalenti di cloruro di sodio (NaCl), sia gli effetti del trattamento nel corso dell'apprendimento, sia infine i fenomeni di tolleranza che si sviluppano con il trattamento ripetuto.

Va notato che, a seguito dei risultati prevalentemente negativi di esperienze preliminari, le prove qui descritte sono state condotte con dosi elevate dei prodotti. Tali esperienze vengono presentate non solo in quanto dimostrano la scarsità degli effetti del MSG, ma anche in quanto costituiscono una ulteriore messa a punto di procedimenti sperimentali per lo studio di eventuali azioni tossiche di vari tipi di prodotti sul comportamento condizionato nell'animale di laboratorio.

Parte sperimentale

Prima dell'inizio delle esperienze sul comportamento condizionato sono state eseguite prove di tossicità acuta con MSG e NaCl per via orale a ratti e topini, secondo il metodo convenzionale.

Per le prove di condizionamento sono stati impiegati ratti maschi albini, allevati nel nostro Istituto, appartenenti ad un ceppo derivato dal Wistar. Gli animali pesavano 200-250 g all'inizio degli esperimenti.

Apparecchiatura

È stata adoperata una serie di apparecchi per lo studio del condizionamento bidirezionale di salvaguardia, con programmazione e registrazione automatica (shuttle-box) (4). Ogni apparecchio comprende una scatola rettangolare di 490 x 270 x 225 mm, connessa ad un sistema di programmazione e ad un registratore di eventi. La scatola, senza alcuna separazione all'interno, ha il pavimento costituito da sbarre di metallo del diametro di 3 mm attraverso le quali può essere somministrato lo shock elettrico (stimolo incondizionato, SI), che in queste esperienze è stato sempre di 1,5 mA. Il pavimento è bilanciato su un perno centrale in modo da permettere a dei microinterruttori di segnalare la presenza dell'animale nella parte sinistra o destra della gabbia. Al centro del soffitto si trova una sorgente luminosa di 10 w che fornisce lo stimolo condizionato (SC).

Programma di condizionamento

Gli animali sono stati sottoposti quotidianamente, salvo la domenica, a sedute di condizionamento di 25 minuti durante ciascuna delle quali ricevevano 50 prove ad intervalli regolari di 30 secondi. Ogni prova iniziava con l'accensione dello SC, seguito, dopo 5 secondi, dallo SI. Una risposta di attraversamento durante i primi 5 secondi della presentazione dello SC (risposta condizionata, RC) poneva termine allo stesso SC e preveniva la somministrazione dello SI; una risposta di attraversamento dopo l'inizio dello SI, poneva termine simultaneamente allo SC e allo SI. L'attraversamento della gabbia durante un intervallo fra prove veniva punito con lo shock elettrico, così che l'animale era costretto a tornare in quella parte della gabbia in cui si era conclusa la prova precedente.

Procedimento sperimentale

a) *Effetti del MSG e del NaCl su animali precedentemente addestrati.* - Per tale esperienza sono stati adoperati esclusivamente dei ratti che nel corso di un prolungato periodo di addestramento avevano raggiunto livelli di prestazione elevati e stabili (RC>80% per tre sedute consecutive). Tutti questi animali avevano ricevuto per via orale, prima di ciascuna seduta di addestramento, un volume di acqua pari a quello di soluzione di MSG o di NaCl successivamente somministrato. Per l'esperienza farmacologica vera e propria quattro gruppi di animali sono stati trattati per via orale 30 minuti prima del test, rispettivamente con MSG 5 g/kg, MSG 10 g/kg, NaCl 1,7 g/kg (dose equivalente a quella di 5 g/kg di MSG) e NaCl 3,4 g/kg (dose equivalente a 10 g/kg di MSG).

b) *Effetti del MSG sull'acquisizione del condizionamento.* - Tale esperienza è stata condotta in due parti: nella prima sono stati confrontati un gruppo trattato quotidianamente, per via orale, 30 minuti prima di ciascuna seduta, con 5 g/kg di MSG, ed un gruppo di controllo trattato con acqua; nella seconda sono stati sottoposti alle prove di addestramento un gruppo trattato con 10 g/kg di MSG ed un altro gruppo di controllo. Ciascun gruppo era formato da 18 animali privi di esperienza precedente; essi sono stati sottoposti alle prove per 8 giorni, con un giorno di intervallo tra le prime 5 sedute e le ultime 3. Inoltre nella seconda parte dell'esperienza (cioè quella riguardante la dose di 10 g/kg), gli animali sono stati sottoposti alle prove per altri 3 giorni, onde studiare gli effetti del cosiddetto cambiamento di stato (14), cioè della somministrazione di MSG al gruppo precedentemente trattato con acqua, e viceversa.

c) *Tolleranza al MSG.* - Il riscontro, nel corso dell'esperienza precedente, di fenomeni di tolleranza al MSG ha richiesto l'esecuzione di un'altra esperienza atta a fornire indicazioni preliminari sui meccanismi responsabili della tolleranza stessa. Infatti esperienze precedenti hanno dimostrato che la scomparsa dell'effetto di uno psicofarmaco sul comportamento a seguito di trattamenti ripetuti può, o meno, essere dovuta ad una tolleranza vera e propria, in quanto può, o meno, verificarsi in animali trattati dopo, anzichè prima di ciascuna prova (6, 5). Pertanto un gruppo di 12 ratti precedentemente addestrati è stato trattato per 12 giorni con acqua prima delle prove e con MSG dopo le prove (5 g/kg al giorno per i primi 6 giorni e 10 g/kg al giorno per gli altri 6 giorni). Successivamente 10 g/kg del prodotto sono stati somministrati 30 minuti prima dell'inizio della prova per 2 giorni. Un altro gruppo di 14 ratti è stato invece trattato soltanto con acqua prima e dopo le prove per 12 giorni e, quindi, con MSG prima delle prove per gli ultimi 2 giorni dell'esperienza.

Risultati

TOSSICITÀ

Le DL50 del MSG e del NaCl sono indicate nella Tabella I.

Effetti acuti del MSG e del NaCl su animali precedentemente addestrati

La Tabella II indica la frequenza delle risposte condizionate di salvaguardia prima e dopo i trattamenti. Appare evidente che solo dosi assai elevate di MSG e di NaCl, cioè superiori alla metà delle rispettive DL50, hanno provocato una riduzione significativa della frequenza delle RC.

TABELLA I

Tossicità acuta del glutammato monosodico (MSG) e del cloruro di sodio (NaCl) somministrati per via orale (*).

	Topino	Ratto
MSG	19,20 (22,84-16,13)	16,60 (18,90-14,50)
NaCl	4,00 (4,38-3,65)	4,20 (4,43-3,98)

(*) DL50 (g/kg) e relativi limiti di fiducia al 95% calcolati secondo il metodo di Litchfield e Wilcoxon (12).

TABELLA II

Effetti del gultammato monosodico (MSG) e del cloruro di sodio (NaCl) sul comportamento di salvaguardia in ratti precedentemente addestrati.

		N.	Media delle RC (in %) ± E.S.			
			Controlli	Trattati	Differenza	p (*)
MSG	5 g/kg	21	77,4 ± 3,2	71,7 ± 4,6	5,7 ± 3,0	n.s.
	10 g/kg	18	86,1 ± 3,4	51,2 ± 6,9	34,9 ± 5,2	<0,001
NaCl	1,7 g/kg	16	90,0 ± 2,0	82,6 ± 4,2	7,4 ± 4,2	n.s.
	3,4 g/kg	17	88,9 ± 1,5	66,1 ± 6,7	22,8 ± 6,5	<0,005

(*) Livelli di significatività (t di Student per osservazioni appaiate, a due code).

Effetti del MSG sull'acquisizione del condizionamento

I risultati della prima parte di tale esperienza non vengono presentati in dettaglio, in quanto non si sono riscontrate differenze degne di nota fra il gruppo di controllo e quello trattato con 5 g/kg di MSG. Ad esempio, la percentuale media di RC lungo tutto l'arco degli otto giorni di prove è stata pari a 40 RC nel gruppo di controllo ed a 37 nel gruppo trattato con MSG.

Nella seconda parte dell'esperienza, condotta con una dose assai più elevata di MSG (cioè somministrando ogni giorno più della metà della DL50) si è osservato un ritardo nell'acquisizione del comportamento di salvaguardia, ritardo tuttavia assai modesto e statisticamente significativo soltanto nella quarta e sesta seduta (Fig. n. 1).

I risultati ottenuti nelle sedute successive a quelle di acquisizione hanno confermato quanto già osservato nelle esperienze precedenti, cioè la significativa riduzione di frequenza delle RC a seguito della sommini-

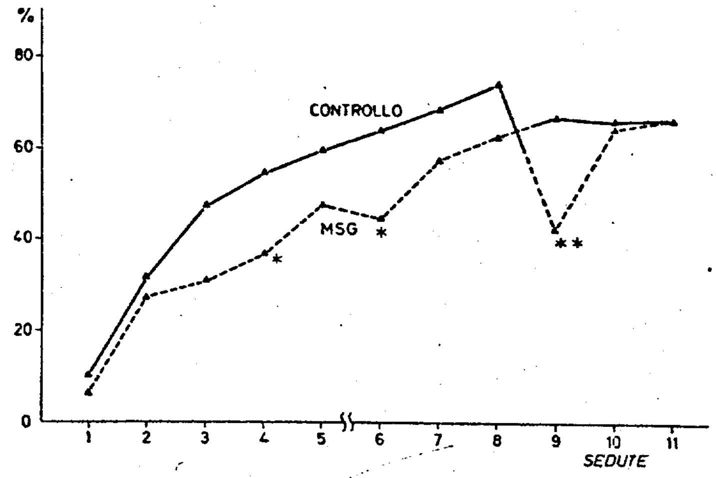

Fig. n. 1

Effetti del glutammato monosodico (MSG 10 g/kg p.o.) *sull'acquisizione del condizionamento di salvaguardia nel ratto.*

In ordinata è indicata la percentuale delle risposte di salvaguardia. Gli asterischi alla 4ª e 6ª seduta indicano differenze statisticamente significative fra controlli e trattati (t di Student a due code per campioni indipendenti, $p<0,05$ e $<0,02$). Dopo l'8ª seduta il trattamento è stato sospeso; inoltre, ai ratti precedentemente adoperati come controlli è stata somministrata la stessa dose di MSG prima di ciascuna delle ultime tre sedute. Il doppio asterisco alla 9ª seduta indica una caduta significativa della frequenza di risposte condizionate nel gruppo precedentemente non trattato (confronto fra 9ª e 8ª seduta, t per osservazioni appaiate, $p<0,005$).

strazione di 10 g/kg di MSG ad animali precedentemente addestrati. Tuttavia la ripetizione di tale trattamento ha condotto in breve ad una scomparsa dell'effetto, dimostrando il rapido instaurarsi di una tolleranza al prodotto. La sospensione della somministrazione di MSG nell'altro gruppo non ha provocato variazioni degne di nota della frequenza di RC.

Tolleranza al MSG

La Fig. n. 2 dimostra chiaramente che gli effetti di una dose elevata di MSG, somministrata 30 minuti prima delle prove (v. gruppo di controllo) si attenuano non solo a seguito della somministrazione ripetuta del prodotto prima delle prove, ma anche a seguito della somministrazione ripetuta del prodotto dopo le prove. Infatti anche se in ambedue i gruppi vi è stata una caduta significativa della frequenza di RC nella prima seduta preceduta da trattamento con MSG, tuttavia tale caduta è stata assai meno marcata nel gruppo che aveva precedentemente ricevuto il prodotto più volte dopo le prove. La differenza fra le cadute os-

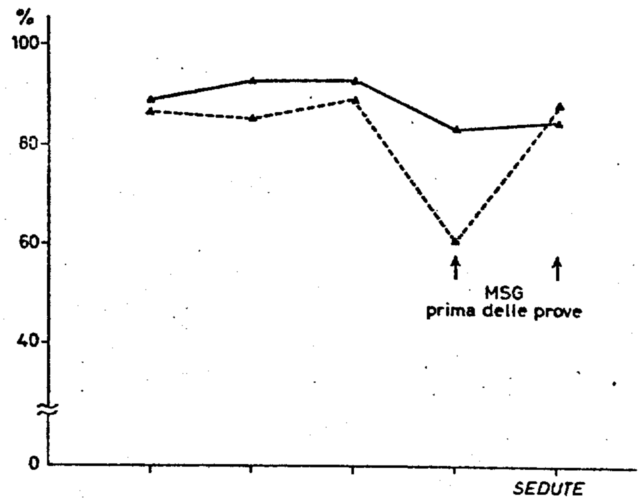

Fig. n. 2

Tolleranza al glutammato monosodico (MSG).

Il MSG (10 g/kg p.o.) è stato somministrato negli ultimi due giorni dell'esperienza 30 minuti prima delle prove a due gruppi di ratti: il primo trattato soltanto con acqua (linea spezzata) per i dodici giorni precedenti, il secondo precedentemente trattato con MSG (linea continua) dopo ciascuna prova (5 g/kg per sei giorni e 10 g/kg per altri sei giorni). Si nota nel secondo gruppo una notevole attenuazione della caduta di frequenza delle risposte condizionate normalmente provocata dalla somministrazione di 10 g/kg di MSG prima della prova. I dati riguardano la percentuale delle risposte condizionate negli ultimi cinque giorni di esperienza.

servate nei due gruppi è risultata statisticamente significativa ($p < 0,02$ con il test non parametrico di Mann-Whitney, utilizzato a causa della presenza di una significativa eterogeneità delle varianze).

Discussione

I risultati delle esperienze sopra riportate indicano chiaramente come gli effetti del MSG sul comportamento condizionato di salvaguardia siano di scarsa entità finchè non si procede alla somministrazione di dosi dotate di azione tossica, cioè superiori alla metà della DL50. Questa conclusione riguarda non solo le esperienze con animali precedentemente addestrati, ma anche quelle in cui il prodotto è stato somministrato durante il periodo dell'apprendimento, onde accrescere la probabilità di mettere in evidenza un'eventuale azione depressiva.

È noto che una caduta delle prestazioni dopo un trattamento psicofarmacologico può essere dovuta non ad un'azione depressiva vera e propria, ma al cosiddetto cambiamento di stato (*change of state*), laddove, in una determinata situazione, la corretta esecuzione dell'esercizio sia dipendente dallo stato (*state-dependent*) (14). In tale caso una caduta delle prestazioni si osserva sia quando si procede alla somministrazione di un prodotto ad animali precedentemente non trattati, sia quando si sospende la somministrazione in un gruppo precedentemente trattato. Questa evenienza sembra esclusa nel caso del MSG: infatti non si è osservata una riduzione della frequenza di RC dopo la sospensione della somministrazione del prodotto in quegli animali che avevano acquisito il comportamento di salvaguardia in condizioni di continuo trattamento.

Va anche notato che nel corso dell'apprendimento non si è osservata un'azione stimolante del prodotto somministrato a dosi sub-tossiche (5 g/kg), a differenza di quanto precedentemente riportato a proposito dell'apprendimento nel labirinto (22). Tuttavia occorre essere più cauti nell'escludere la presenza di un'azione facilitante, che spesso compare soltanto in test o condizioni assai particolari, che non nell'escludere la presenza di un'azione depressiva, rispetto alla quale vari test forniscono risultati relativamente meno eterogenei.

Va inoltre notato che, già alla seconda somministrazione, la stessa dose più elevata di MSG (10 g/kg) perde una buona parte del suo effetto. È noto che fenomeni di questo tipo possono essere dovuti a cause diverse, cioè sia ad una tolleranza nel senso classico della parola — come nel caso dei narcotici e dell'LSD-25 — sia all'entrata in funzione di sistemi di compenso che correggono il deficit di comportamento senza che (o prima che) si sia sviluppata una vera e propria tolleranza. Quest'ultima evenienza è stata più volte dimostrata nel corso di trattamenti ripetuti con amfetamina (18) o scopolamina (5, 8, 2). Una distinzione fra i due tipi di desensibilizzazione viene generalmente eseguita mediante apposite esperienze, cioè: a) quelle che mettono, o meno, in evidenza notevoli differenze di velocità di desensibilizzazione da un test all'altro; b) quelle che mettono, o meno, in evidenza notevoli differenze di velocità di desensibilizzazione a seconda che i trattamenti ripetuti vengano effettuati prima di ogni seduta di prove o in altro momento, in genere dopo la fine di ciascuna seduta. Nel caso del MSG è apparso evidente che il trattamento ripetuto dopo ciascuna prova, ha notevolmente ridotto gli effetti del prodotto al momento in cui si è iniziata la sua somministrazione prima delle prove. Pertanto si deve concludere che l'evidenza è a favore del rapido sviluppo di una vera e propria tolleranza.

Questo fenomeno tende d'altra parte a sminuire il significato dei risultati essenzialmente negativi ottenuti nelle esperienze in cui il prodotto è stato somministrato sin dall'inizio dell'apprendimento (assenza di azione a 5 g/kg, azione depressiva poco marcata a 10 g/kg). Infatti al primo giorno le prestazioni sono sempre assai basse anche nei gruppi di controllo, il che lascia poco spazio per la dimostrazione di un ritardo nell'apprendimento (cosiddetto *floor effect*). Nei giorni successivi la mancanza o scarsezza di effetti depressivi potrebbe essere attribuita al rapido instaurarsi di una tolleranza.

Occorre infine ricordare che, anche per la scarsezza di dati in letteratura, tali risultati essenzialmente negativi non si prestano, al momento attuale, ad estrapolazioni e a generalizzazioni ad altre situazioni o ad altre specie. In particolare, come è stato già ricordato all'inizio, l'elevazione anche notevole dei tassi ematici di acido glutammico non si accompagna in animali adulti ad un aumento della concentrazione dell'aminoacido nel cervello (11). Pertanto il reperto negativo ottenuto mediante prove di comportamento nell'animale adulto non è, di per sè, in contrasto con quello di danni arrecati al sistema nervoso centrale dal MSG, se somministrato durante il periodo dello svilpppo (13, 19, 15). Ovviamente rimane da determinare se questi ultimi effetti siano da attribuirsi ad una maggiore sensibilità del SNC nelle fasi di sviluppo e di organizzazione o semplicemente ad una più efficace penetrazione del prodotto attraverso la barriera emato-encefalica.

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Effect of Dietary Monosodium L-Glutamate on Some Brain and Liver Metabolites in Rats (35930)

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The production of acute neuronal degeneration in the retina of mice receiving parenterally administered monosodium L-glutamate (MSG) has been described (1, 2). Brain lesions in the hypothalamus of rat, monkey, and mouse have also been associated with administration of MSG by the subcutaneous route (3–5) and in the mouse by the oral route (6). The results are in disagreement with the findings of Adamo and Ratner (7) who were unable to demonstrate any effects on brain or reproduction function in rats.

Glutamic acid is highly concentrated in nervous tissue and plays a significant role in its metabolism (8). Although no net uptake of glutamic acid in brain can be demonstrated after excessive elevation of blood glutamic acid (9, 10), the glutamic acid from blood exchanges rapidly with that from brain (11, 12). The present study is an attempt to establish a biochemical basis for the action of MSG relating to its metabolic function as an energy source in brain and as a precursor for biologically active metabolites in brain and liver.

Materials and Methods. MSG was purchased from Sigma Chemical Company. L-Glutamic acid-U-^{14}C (197 μCi/μmole) used in the *in vitro* incubation study was obtained from the New England Nuclear Corporation. Purina laboratory chow was purchased from Ralston Purina Company.

Fifty male Holtzman albino weanling rats were allotted to five groups of 10 animals each and were housed individually. The diets, Purina laboratory chow supplemented with MSG at the 0, 1, 5, 10, and 20% levels, were fed *ad libitum* for 16 weeks. Body weights were recorded weekly. At the end of the experimental period the rats were lightly anesthetized with ether and decapitated. Whole brain was rapidly excised and weighed, and a 16% homogenate was prepared in 0.1 *M* phosphate buffer (pH 6.1) for assay of glutamic acid decarboxylase (GAD), the enzyme that may be the rate-limiting step in determining the level of γ-aminobutyric acid (GABA) in particular areas of the central nervous system (13). For the assay of GAD, the final reaction mixture of 2.2 ml contained 1.4 ml of phosphate buffer (pH 6.1 at 36°), 0.2 ml of homogenate, 0.2 ml of pyridoxal phosphate (500 μg/ml), 0.1 ml of MSG (500 μmole), and 0.3 ml of MSG-^{14}C (2.2×10^6 cpm and 0.0076 μmole). The mixture was shaken at 36° in a constant-temperature water bath for 10 min; aliquots were withdrawn at 2.5 min intervals during the incubation period and added to tubes containing 1.3 vol of cold 0.6 *N* perchloric acid. After the tubes were centrifuged the supernatant was removed, neutralized, and reduced in volume under vacuum. An appropriate volume was applied to paper strips in a Durrum cell for electrophoresis in a pyridine:acetic acid:water (8:15:977) system at 300 mV for 3 hr. Areas on the paper corresponding to those of authentic GABA were cut out, placed in a vial containing 15 ml of Bray's solution (14), shaken mechanically for 30 min, and counted for radioactivity in a Nuclear Chicago Mark 1 liquid scintillation counter. Measurements were corrected for quenching as previously described (15). The remainder of the brain homogenate was saved for colorimetric assay of protein (16) and DNA (17). Glutamate (18), GABA (19), and aspartate (20) concentrations in protein-free brain extracts were determined by enzymatic methods. Glutamine was converted to glutamate with the enzyme glutaminase and assayed as described above. Succinate was

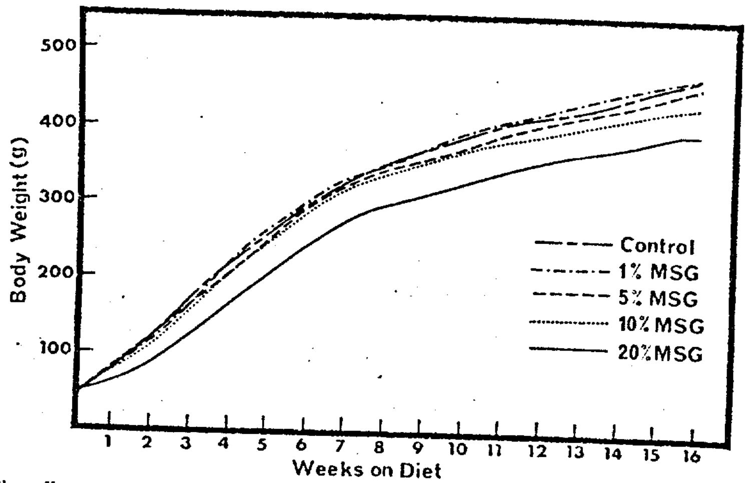

FIG. 1. The effect on body weight of feeding various levels of MSG to rats for 16 weeks.

assayed by a previously described enzymatic method (21) after its isolation by eluting from Dowex-1-formate columns (22).

Whole liver, removed at the time of sacrifice, was frozen in Freon and weighed, and sections were taken for protein, RNA (15), and DNA determinations. The remainder of the liver was acid extracted (22), and the supernatant fraction was assayed for glutamate, lactate (23), malate (24), aspartate, and α-glycerophosphate (α-GPO_4) (25).

Data were analyzed by the Student *t* test.

Results. Figure 1 shows that rats fed the 20% MSG-supplemented diet did not grow as well as control rats or rats fed the chow diet supplemented with MSG at the 1, 5, or 10% levels. By week 16, body weights of rats fed the 20% MSG diet were depressed by 15% compared with those of control rats ($p<.001$).

The data in Table I show some of the effects of feeding MSG on its metabolites in rat brain. GABA concentrations in brains of rats fed the 1% MSG diet were decreased by 17% from control values ($p<.025$) with a maximum decrease of 20% in rats receiving the highest dietary level of MSG ($p<.005$). Hyperirritability, an excessive response to handling, was observed in all groups of rats consuming diets supplemented with MSG. Succinate concentrations in brain were elevated as dietary levels of MSG were increased; in rats fed the highest level of MSG, the succinate was increased 20% compared to control values ($p<.05$). No changes were noted in brain weight, protein, or DNA. Despite the high MSG intakes, brain glutamate remained relatively constant as did GAD, the enzyme responsible for the formation of GABA. Glutamine and aspartate concentrations in brain were also unresponsive to dietary levels of MSG.

Table II shows some of the effects of feeding MSG on metabolites in rat liver. The liver weights of rats fed the 20% MSG diet were depressed by 9% ($p<.025$); however, liver:body weight ratio was not significantly different from that of controls. Aspartate concentrations in all rats receiving MSG were elevated compared with those of controls; the maximum elevation was 25% in rats given the highest level of dietary MSG ($p<.025$). No significant changes were observed in liver protein, RNA-P, or DNA-P. Liver glutamate and α-GPO_4 remained unchanged, although an upward trend in their concentrations was observed. Lactate and malate levels were not significantly changed in liver.

Discussion. Glutamic acid in the form of the sodium salt is widely used as a seasoner or flavor enhancer for many foods, and as a drug in the treatment of ammonia accumulation in hepatic failure and various behavioral aberrations (26, 27). Glutamate enters the brain rapidly (11, 12) and is quickly metabolized to other substances; there was no net increase in brain glutamate levels of rats ingesting the 20% MSG diet. Figure 2 shows that increased concentrations of brain succinate can be derived from MSG by two

TABLE I. The Effects of Various Dietary Levels of MSG on Some Biochemical Parameters of Rat Brain.[a]

Dietary MSG (%)	Brain wt (g)	Protein (mg/g)	GAD[b]	DNA (mg/g)	Glutamate (μmole/g)	GABA (μmole/g)	Glutamine (μmole/g)	Aspartate (μmole/g)	Succinate[c] (μmole/g)
Control	2.01 ±0.05	114 ±3	69 ±8	1.18 ±0.05	9.20 ±0.56	2.59 ±0.10	1.12 ±0.20	2.43 ±0.09	0.401 ±0.033
1	1.97 ±0.05	118 ±3	64 ±5	1.25 ±0.04	9.38 ±0.34	2.16[d] ±0.11	1.37 ±0.22	2.30 ±0.18	0.467 ±0.055
5	1.89[e] ±0.01	117 ±1	70 ±7	1.28 ±0.05	9.38 ±0.08	2.11 ±0.31	1.21 ±0.18	2.38 ±0.03	0.460[e] ±0.008
10	1.90 ±0.07	116 ±3	61 ±5	1.19 ±0.07	9.78 ±0.27	2.20[f] ±0.08	1.72 ±0.15	2.41 ±0.10	0.503 ±0.063
20	1.92 ±0.03	116 ±1	64 ±5	1.30 ±0.07	9.25 ±0.18	2.07[g] ±0.08	1.07 ±0.28	2.31 ±0.05	0.508[e] ±0.030

[a] Each value is the mean of 8 to 10 animals and is given with the SE.
[b] Glutamic decarboxylase activities (μmoles of GABA formed/g of brain/hr).
[c] These values represent three brain extracts; each extract was derived from two brains.
[d] Significantly different from control, $p < .025$; [e] $p < .05$; [f] $p < .01$; [g] $p < .005$.

TABLE II. The Effects of Various Dietary Levels of MSG on Some Biochemical Parameters of Rat Liver.[a]

Dietary MSG (%)	Liver wt (g)	(mg/g)			(μmole/g)				
		Protein	RNA-P	DNA-P	Glutamate	Aspartate	Lactate	Malate	α-GPO_4
Control	16.56 ±0.56	202 ±3	0.699 ±0.017	0.106 ±0.006	1.58 ±0.10	0.44 ±0.02	11.25 ±0.49	0.53 ±0.03	0.94 ±0.05
1	16.46 ±0.40	194 ±2	0.699 ±0.024	0.118 ±0.005	1.51 ±0.07	0.53[b] ±0.03	10.80 ±0.51	0.58 ±0.03	0.96 ±0.03
5	16.85 ±1.01	194 ±5	0.701 ±0.028	0.110 ±0.006	1.64 ±0.06	0.54[b] ±0.02	11.33 ±0.56	0.59 ±0.03	1.04 ±0.11
10	15.93 ±0.55	198 ±4	0.719 ±0.028	0.118 ±0.007	1.70 ±0.11	0.56[c] ±0.05	10.75 ±0.45	0.57 ±0.03	1.22 ±0.13
20	15.02[b] ±0.28	200 ±2	0.729 ±0.017	0.107 ±0.006	1.76 ±0.16	0.58[b] ±0.03	11.28 ±0.59	0.59 ±0.03	1.18 ±0.11

[a] Each value is the mean of eight to ten animals and is given with the SE.
[b] Significantly different from control: $p < .025$; [c] $p < .05$.

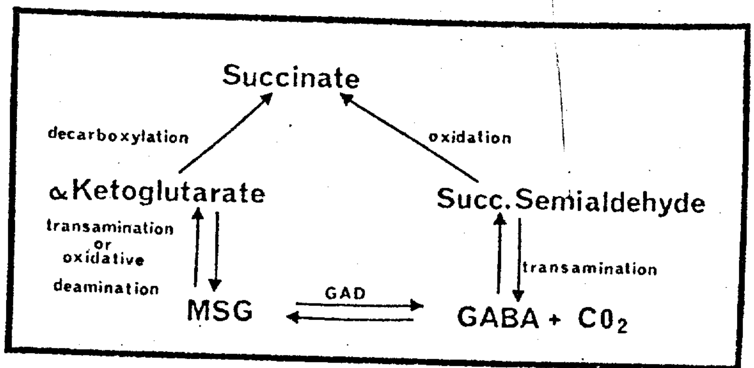

FIG. 2. The metabolic pathway of MSG in the rat brain.

widely accepted reactions: (a) oxidative deamination or transamination of glutamate to α-ketoglutarate, which is further decarboxylated; and (b) transamination and oxidation of GABA, a decarboxylation product of glutamate. However, the most important finding is the decreased concentrations of GABA in the brains of rats in all groups fed MSG diets. These rats displayed increased irritability to a type similar to that seen in vitamin B_6 deficiency. In the type of irritability induced by vitamin B_6 deficiency or isoniazid treatment, GAD activity is decreased because of insufficient coenzyme, resulting in decreased production of GABA. Supplementation with the vitamin reversed this effect (28, 29). In our experiments, decreased concentrations of brain GABA were not associated with a vitamin B_6 deficiency or with GAD activity (added vitamin B_6 did not increase enzyme activity in the assay), which remained constant in all groups of rats. It is possible that an initial and transient increase in GABA production stimulated those enzymes responsible for its degradation.

Decreased protein and DNA concentrations in brain have been associated with neuron deficiency (30); however, in our studies, these parameters remained constant in rats ingesting MSG, indicating that there was no impairment in brain development.

The liver also metabolizes glutamate at a rapid rate, as shown by the consistent values for glutamate in the livers of rats fed MSG in the diet, even at the highest level. Aspartic acid, which was elevated in the livers of MSG-fed rats, was probably derived from the main degradative pathway of glutamate, transamination with oxaloacetate. Oxidative pathways for the production of energy, CO_2, and water by way of the citric acid cycle were also unaffected by dietary MSG; levels of lactate, malate, and α-GPO_4 in the liver remain constant.

Summary. Rats in each group receiving MSG exhibited hyperirritability. While levels of glutamate, glutamine, aspartate, DNA, protein, and GAD in brain remained relatively constant, a significant decrease in GABA levels was noted along with a significant increase in succinate levels. Analysis of liver indicated no effect of dietary MSG on protein, RNA-P, DNA-P, glutamate, lactate, malate, or α-GPO_4. Aspartate levels were significantly increased. The decrease in brain GABA levels may be related to the observed increased irritability.

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BEET SENSITIVITY: ALLERGIC REACTIONS FROM THE INGESTION OF BEET SUGAR (SUCROSE) AND MONOSODIUM GLUTAMATE OF BEET ORIGIN

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DUKE[1] was the first to describe sensitivity to beet sugar although sensitivity to beets as a vegetable has long been recognized. In more recent years numerous cases of beet sensitivity in which the ingestion of beet sugar has resulted in allergic symptoms have been noted also by Rinkel[2] and Zeller.[3]

The fact that other crystalline sugars[4-6] are known to produce allergic symptoms warrants an investigation of the probable allergenicity of beet sugar. Another factor favoring such a study is the relative frequency with which beet sugar is used, for it is generally known that the incidence of specific sensitivity to foods is closely related to the incidence of such foods in the dietary.[7]

Beet sugar is the principal source of granulated sugar used in certain wide geographic regions of this country, particularly in the areas in which beet sugar mills are located and in regions relatively far removed from cane refineries or otherwise inaccessible to water transportation therefrom. In general this would include the entire western half of the United States and to some extent the north central states, particularly Michigan, Minnesota, and Wisconsin.

In recent years beet sugar has been favored by manufacturers of processed foods not only in the regions mentioned but elsewhere in view of the favorable price differential as compared with cane sugar. The current widespread use of beet sugar in food processing makes it probable that most individuals in this country are receiving some beet sugar daily, frequently several times daily.

Another source of exposure to beet is the rapidly increasing use of monosodium glutamate,[8] a so-called chemical condiment derived either from beets, wheat, corn or soy bean, incorporated into many processed foods as well as being added directly to various foodstuffs in their preparation in public eating places as well as in the home. It is most commonly employed to accentuate meat flavors in soups, chowders, and bouillons, sauces, gravies, canapés, salads, creamed dishes, and cheese dishes and is added directly to vegetables, meats, poultry, game, and fish.

The detection of the presence of a specific monosodium glutamate in a prepared food is exceedingly difficult because of the reluctance of cooks to

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admit that they have used such an agent and the difficulty to ascertain whether the product used might have been derived from beets or one of the other sources from which this material is made. The latter is particularly true in the usual absence of specific food sources on the labels of these commercial products.

CASE REPORTS

The following are examples of beet sensitivity.

F. A., a college mathematics professor, aged 43, had been subject to perennial nasal allergy since adolescence. During the past fifteen years he had also complained of chronic fatigue and intermittent bilateral frontal headaches. The fatigue syndrome in his case was characterized by mental confusion, impairment of memory, and a certain degree of aphasia which materially interfered with his teaching in the mid- and late afternoons. In addition he had also been subject to troublesome backaches for the past decade which usually awakened him between two and four in the morning. Superimposed on chronic symptoms of this type were acute episodes of very severe cramping pains in the leg muscles to the point that it was exceedingly difficult ot walk. In 1945 he was diagnosed as having a ruptured intervertebral disc for which a spinal fusion was performed. Although the back symptoms were improved following this operation, he reported accentuation of his residual chronic back and leg symptoms coincident with each recurrence of soreness, scratchiness, and irritation of his throat. Beginning a few months prior to the operation he also reported drawing, pulling pains across the upper back and shoulders and bouts of acute torticollis on a few occasions. He commonly reported feeling sore and stiff in all muscles on awakening in the morning.

He was found to be dust sensitive on the basis of intradermal tests, but dust therapy failed to alter the course of the symptoms. There was no other evidence of inhalant sensitivity.

During the third day of wheat avoidance in preparation for an individual food test with wheat he reported an improvement in the nasal symptoms and fatigue; the experimental feeding of wheat was followed by the development of a frontal headache associated with pulling, drawing, and tautness of the posterior cervical muscles and the delayed development of an acute lower backache. Tests with corn and eggs were followed by similar but less severe symptoms. Test ingestion of lettuce was followed by the development of a headache three hours after the meal. Potatoes were associated with the precipitation of a severe headache, abdominal pains, and excessive belching. Reactions of lesser degree followed the individual test ingestion of oats, rice, pork, orange, and tomatoes. Tests with several other major foods were compatible. At this point the chronic symptoms were materially relieved but the patient still complained of localized and generalized muscle symptoms, some fatigue, and mental symptoms.

In checking his food diary it was learned that he had awakened two or three times nightly with backache whenever he had eaten beets; this agreed with the childhood history of vomiting after spinach and severe headaches following the ingestion of Swiss chard, members of the same botanical family.

He was prepared for an individual food test with beet sugar by the avoidance of all beets and beet sugar for a period of four days prior to the experimental ingestion of 75 Gm. of beet sugar. Fifty minutes later he reported the gradual onset of pressure in the frontal area of the head, tautness of the posterior cervical muscles, and heartburn. As the headache became accentuated he developed severe abdominal cramps and flushing of the face. He remained unusually fatigued for the following two days. With the avoidance of beets and commercially sweetened foods containing beet sugar he reported marked and sustained improvement in the remainder of the chronic symptoms. The patient slept more soundly at night and was able to sleep on his abdomen for the first time since the spinal fusion. He noted that he was much less restless than formerly, being able to sit in his chair for long periods of time without discomfort or the necessity of frequently changing his position, that he had what he considered a normal amount of energy, and that he was much more alert mentally, which reflected itself in greater degrees of lucidity and ease of expression than he had enjoyed in several years. At the time of his last visit, a year after the diagnosis of beet sensitivity, he was actively engaged in a research project in addition to his full-time teaching position.

The patient remained highly sensitive to beets, either in the forms of beets as such or beet sugar. On several occasions the accidental ingestion of beet sugar was followed by a precipitation of fatigue, headache, myalgia, and mental confusion.

At this time he volunteered for testing the effect of monosodium glutamate, a chemical condiment prepared from Steffen's waste, a beet residue following the extraction of the sugar. After the ingestion of one teaspoon of the powder dissolved in a glass of water, he developed a frontal headache which became progressively more severe for two hours. The following morning he awakened with marked stiffness of the neck, shoulders, and lower back and remained lame, stiff, and tired for the rest of that day. He noticed residual fatigue and mental confusion the second day and then remained symptom free. A control test with monosodium glutamate prepared by the same company* but derived from wheat was not associated with reactive symptoms. At the time of this test, wheat had been excluded from the diet for a year and the patient was known to have a tolerance for an occasional feeding of wheat. The patient was unaware whether he was receiving the wheat or the beet preparation at the time of the tests.

R. C., a housewife, aged 42, had been subject to intermittent hives for the past ten years prior to her initial visit in March, 1948. Although she had had attacks of urticaria at any time of the year, her symptoms were much more troublesome from the fourth of July to Labor Day. In the last year, however, this seasonal incidence had changed and she had continued to have daily chronic urticaria throughout the winter.

She had known that the ingestion of clams was followed by pruritus and swelling of the buccal mucous membranes, nasal stuffiness, and lacrimation. Other foods were not suspected.

There were no abnormalities of the physical examination except for the typical urticarial plaque lesions. Skin tests with inhalants were negative except for a borderline reaction to house dust, but specific therapy with dust failed to alter the course of the symptoms.

The patient was studied for specific food allergy by means of individual food tests. The experimental ingestion of wheat, corn, milk, egg, and tomato was not associated with reactive symptoms. A similar test with potato was followed by the development of a severe headache beginning ten minutes after the initial feeding and became associated with marked fatigue and pulling and drawing sensations of the posterior cervical muscles. One hour after the test ingestion of coffee the patient developed tenseness and nausea, followed shortly by the onset of severe generalized urticaria which persisted for four days, whereas for a few days prior to this test she had been having only mild, intermittent hives.

Although the avoidance of potato and coffee were helpful, this plan did not relieve the chronic urticaria and neither did various elimination diets tried successively at this juncture.

The additional history then became available that the hives had been present only since the patient began spending the summers in Michigan, and until the past year the hives had subsided upon her return to Chicago. This immediately aroused the suspicion of beet sensitivity inasmuch as this appears to be more common in Michigan than in Illinois because of the wider use of beet sugar in Michigan. Then the additional pertinent fact came to light that during the war she had bought a large supply of Michigan beet sugar and had used it exclusively each summer. Furthermore, in returning to Chicago in the fall of 1947 she had brought the remainder of this sugar with her and had continued using from it for several months. Upon the exhaustion of this source of supply she had purchased additional beet sugar in the local market.

The patient was tested with beets and beet sugar after four days of avoidance and developed a sharp recurrence of urticaria one-half hour following the second feeding which persisted for the following two days.

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*Samples were furnished through the courtesy of International Minerals and Chemical Corporation, Chicago, Ill.

She then remained free of urticaria and other allergic symptoms except for an occasional recurrence following meals away from her own home. However, continual urticaria again developed in the spring of 1949 coincident with the onset of the golf season. She played golf on the average of four or five days a week and always drank an "old fashioned" after her game. It then occurred to her to investigate the type of sugar used in the bar. Finding that beet sugar had been used exclusively, henceforth she had her drinks made with cane sugar and the urticaria ceased at the end of three days. She also learned from experience that she was able to tolerate the bakery products from one neighborhood bakery and not from another; inquiry led to the fact that cane sugar was used routinely in the former and beet sugar in the latter.

Upon another occasion she was fed experimentally one-half teaspoonful of monosodium glutamate of beet origin in a half glass of water (at the time of the test she was unaware whether she was receiving monosodium glutamate of beet, wheat, or corn origin). Ten minutes later she noticed a sense of pressure in her temples, followed shortly by extreme weakness and faintness which persisted for an hour. Intense erythema and pruritus of the skin about the eyes, anterior neck, and upper back developed at eighty minutes; scratching was followed by urticarial lesions. At the end of two hours the patient was observed to have generalized urticaria of the entire upper part of the body; this persisted and spread to involve the entire body during the night of the test. Three days were required for this attack to subside. A similar amount of monosodium glutamate of corn* and of wheat origin was not followed by urticaria or other symptoms.

L. T., a housewife, aged 32, had been subject to intermittent eczema and ragweed hay fever since childhood; the latter had been complicated by seasonal bronchial asthma in recent years. She also complained of intermittent stiffness and aching of the posterior neck muscles, frequently followed by dizziness and generalized headaches. There had also been intermittent bouts of burning and smarting on urination, swelling and soreness of the knees and finger joints, and a chronic degree of fatigue, all of which had failed to respond to various types of treatment

On examination the patient had an erythematous scaly eruption of several fingers, a fusiform enlargement of the mid-finger of her right hand with edema and discoloration of the skin of the infraorbital areas.

She was found highly sensitive to corn, wheat, and beets on the basis of individual food tests; the corn reaction consisted of excessive gas and belching with pronounced clearing of the throat beginning at forty minutes, followed by flushing of the face, somnolence, and the development of a severe headache. The wheat test was followed by the development of transitory tightness of the chest, somnolence, and marked flushing of the face beginning twenty minutes after the initial feeding. Difficulty in focusing her eyes, extreme fatigue, and nervousness persisted for the remainder of the day. Beet sensitivity was suspected on the basis of an acute reaction following the ingestion of beets as determined from evidence from her food diary and the fact that she had been using beet sugar exclusively for the past two years. An individual food test with beets was followed by somnolence beginning at twenty minutes and a severe frontal and occipital headache at sixty minutes which persisted for the remainder of the day. The following morning she passed several diarrheic stools and there was a definite flare of the dermatitis of her hands, they being more pruritic and erythematous than any time in several recent days. The patient also developed a port wine colored urine the morning after eating beets.

She was then fed 50 Gm. of beet sugar after not having eaten any type of beet product for several days; this was followed by sleepiness at five minutes which interfered with her ability to read comprehendingly. She complained of feeling nervous and tense and particularly of restlessness of her legs. Associated with the leg symptoms was a sensation of tightness and stiffness of the knee joints and the desire alternately to flex and extend the knees.

*Furnished through the courtesy of A. E. Staley Mfg. Co., Decatur, Ill.

With the complete avoidance of beets the patient noticed relief of her formerly troublesome arthritis in that she remained free of the stiffness and soreness of her knees and finger joints and was able to move them painlessly through their full range of motion.

The dermatitis of her hands flared from contact with cornstarch in ironing or with wheat flour in the course of baking. It is interesting, however, that she was able to iron without an accentuation of the dermatitis if she used arrowroot starch in place of cornstarch or if she only inhaled the fumes of baking wheat containing products and avoided direct contact of her hands with uncooked flour.

Because of the exceedingly high degree of specific sensitivity to corn and beets in this patient and the fact that the ingestion of either corn sugar (glucose or dextrose) or beet sugar (sucrose) caused an immediate accentuation of her symptoms, she was fed experimentally 50.0 Gm. of alpha D glucose* prepared from beet sucrose with the understanding that the source material was cane sugar. As she had tolerated the ingestion of cane sugar over a period of many months without any suspicion of specific sensitivity, she did not expect a reaction from this test. Much to her surprise, however, she developed a dull frontal headache within five minutes after the second serving of 25.0 Gm. of this material. Within the following hour her headache became progressively more severe and within the second hour she developed anorexia, nausea, and considerable bloating. She remained extremely fatigued and "generally upset" for the following two days. The type of reaction that she experienced prompted her to accuse us of feeding her beet sugar.

On another occasion she was fed experimentally an "unknown" preparation having a salty taste, actually one-half teaspoonful of monosodium glutamate of beet origin in one-half glass of water. She complained of itching of the anterior neck and upper chest between eight and ten minutes after the test feeding. The pruritus spread rapidly to involve the entire neck, being particularly intense about the hair line, and the sites of the previous dermatitis of her fingers. Coincident with the onset of the pruritus she complained of a sharp pressure in the frontal and occipital areas of her head followed immediately by an intense, throbbing, generalized headache. At fifteen minutes she noted an inability to focus her eyes and for the following half hour was unable to read or to see objects clearly. She complained of mild abdominal cramps and nausea beginning at forty-five minutes which persisted for the following ten minutes. The intense headache as well as the blurring of vision had subsided sufficiently at sixty minutes that she was able to read ordinary print. She remained subject to palpitation on exertion and extreme fatigue for the remainder of the day. In retrospect, she likened the early phase of this reaction to the constitutional symptoms she had previously experienced from hay fever therapy, making the statement, "You know, it was the same type of immediate reaction that I get from a hay fever shot that subsequently developed into a severe reaction."

With the avoidance of incriminated foods the patient has had complete relief of her formerly troublesome allergic symptoms with the exception of ragweed hay fever. She refused specific therapy with ragweed extract in view of the repeated severe constitutional reactions that she had formerly experienced in this connection. She also refused to be tested with further unknown solutions and then specifically with monosodium glutamate of wheat and of corn origin when the nature of these proposed tests were explained to her. It is of interest that the detection of beet sensitivity and the avoidance of foods containing beet sugar completed her specific food diagnosis.

M. A., a 38 year-old woman, had been subject to chronic nasal symptoms, intermittent canker sores, and frequent severe incapacitating headaches all her life. The headaches were generalized in location, associated with pulling, drawing, and marked tenderness of the posterior cervical muscles, and commonly with nausea and vomiting. In addition she remained subject to marked fatigue, considerable irritability and nervousness, and a dull constant type of headache persisting between the more acute and incapacitating episodes of head pain.

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*Prepared through the courtesy of Dr. Robert C. Hockett, Research Director, Sugar Research Foundation, New York City, N. Y.

Although she had suspected several minor foods of bringing on headaches and other allergic symptoms, their complete avoidance had failed to bring about a significant degree of relief.

When first seen in 1947 she was found to be moderately dust sensitive, but each of three attempts to measure her degree of house dust allergy by testing with serial intradermal doses in accordance with a previously described technique[9, 10] resulted in immediate nasal stuffiness followed in one half hour by the development of a headache. House dust therapy at very low levels was moderately effective in controlling her nasal symptoms but did not change the course of the headaches, fatigue, or myalgia.

As a result of performing individual food tests with several major foods she was found highly sensitive to potato, developing a very severe headache immediately following this test.

On a program of dust avoidance, specific low level therapy with house dust extract and the elimination of potatoes and other minor incriminated foods she remained free of troublesome symptoms until April, 1949. At this time she developed an attack of bronchitis after massive dust exposure. Intractable coughing persisted during the following four months in spite of repeated efforts to diagnose specific allergic reactions and various types of medications, including oft-repeated doses of Demerol and other opiates. The patient frequently lost consciousness in the more violent attack of coughing.

All sources of beet and cane were avoided simultaneously in preparation for individual food tests. Her coughing ceased for the first time in several months for twenty-four hours prior to the beet test. Eighteen minutes after the ingestion of 20 Gm. of granulated beet sugar the patient developed a severe paroxysm of coughing associated with generalized chilling and followed by the onset of nausea and a very severe headache. The coughing became progressively more severe during the following half hour and she passed into a state of semiconsciousness. Although it was possible to arouse her from the stupor, she subsequently had no recollection of the experience. Intractable coughing and headache persisted for the following three days and required massive doses of opiates and antihistaminics for partial relief. A similar but somewhat less severe clinical reaction followed the experimental ingestion of cane sugar.

With the avoidance of beet and cane in addition to other foods, the patient remained free of headaches and coughing for the following week.

She was then discharged from the hospital during the height of the ragweed pollenating season and drove a distance of 150 miles to her home. Midway on this trip she developed her initial attack of ragweed pollinosis and for the following week continued to have very severe hay fever although she remained relatively free of coughing and headache. She was then rehospitalized, and according to attendant nurses, sneezed, sniffled, or coughed a total of 640 times in a twenty-four hour period prior to the administration of adrenocorticotropic hormone (ACTH, Armour). This aspect of her case has been reported elsewhere.[11] Suffice it to say that the hay fever and all other allergic symptoms completely subsided within twenty-four hours after the patient received the first dose of ACTH. She received a total of 125.0 mg. in five divided doses given at six hour intervals.

Two weeks following the initial beet test, at a time when the patient was completely free of allergic symptoms, an individual food test with beet sugar was repeated and was not associated with reactive symptoms. The cane test was repeated similarly and tolerated. She was then returned to a general diet and continued on such for the following four months without developing any of her formerly troublesome allergic manifestations. The only other medication that the patient received during this interim was a continuation of the maintenance dose of estrogen that she had been receiving for many months prior to the diagnostic allergy studies and initial course of ACTH. However, this patient had a recurrence of allergic manifestations following massive dust exposure four months after the initial therapy with ACTH and again responded with symptomatic relief after additional therapy with ACTH.

DISCUSSION

Beet sensitivity is not an uncommon cause of chronic allergic symptoms. Although there is some reason to believe that beet allergy occurs most frequently in areas where beet sugar is used predominately, it should be pointed out that the use of beet sugar is more widespread than is commonly believed as it is employed in the preparation of commercially processed foods which have a national market. It is significant that beet sugar carries sufficient allergenicity to cause symptoms in many beet-sensitive patients.

In addition to beets as a vegetable and sugar derived from beets, another source of exposure to beet is the use of monosodium glutamate employed as a condiment or seasoning agent, some of which is derived from the protein-containing waste of beet sugar mills. A similar product from the chemical standpoint is manufactured from wheat, another from corn, and probably a smaller amount from soy beans. Samples of monosodium glutamate of beet, corn, and wheat origin have been shown to cause allergic symptoms in at least certain patients possessing high degrees of specific sensitivity to these foods. The amount of wheat monosodium glutamate ordinarily added to a steak has been incriminated as a cause of allergic symptoms in a few wheat-sensitive individuals. A steak of similar size and eaten under similar test conditions but prepared without the addition of wheat monosodium glutamate has not been followed by reactive symptoms in these individuals. Thus far we have not encountered detectable clinical reactions in beet-sensitive patients from the amount of monosodium glutamate of beet origin ordinarily added to a steak in the process of preparing it. Neither have 900 mg. of beet monosodium glutamate, the amount which may be added to a steak for instance, been followed by detectable symptoms when this amount is deliberately added to meat and fed "blindly" to a known beet-sensitive individual. This amount has recently been tested in three additional highly beet-sensitive individuals. However, as there is no doubt that larger amounts of monosodium glutamate of beet origin are capable of eliciting detectable reactions when added to water and ingested by certain beet-sensitive patients, the possibility that accidental exposures of this type in smaller amounts might be a cause of symptoms in certain individuals should be kept in mind.

The study of this problem has been handicapped by the failure of manufacturers to indicate the source materials employed in the manufacture of monosodium glutamate. Until this question can be answered, the absolute avoidance of beets in preparation for individual food tests should attempt to eliminate this possible source of contamination. The same holds for the avoidance of wheat, corn, and soy beans.

Similarly, the failure of federal labeling regulations to differentiate the types of sugar employed in processed foods, that is whether it is of beet, cane, or corn origin, is a further serious handicap to the allergist engaged in the problem of making specific food diagnoses. It is a particularly vexing problem for the allergic individual who happens to be sensitive to one or more of these specific sugars when he is unable to determine by the information on the label of a given product whether it contains one or more of these particular sugars which he is attempting to avoid. This point of view has recently been presented before the Public Hearings of the Food and Drug Administration in respect to the Proposed Standards of Identity of Breads and Related Foods.[12]

Likewise, the utter lack of labeling regulations pertaining to the declaration of the specific food agents employed in the manufacture of various types of alcoholic spirits entails further difficulties for the beet-sensitive patient in view of the widespread preference for beet sugar in the preparation of liquors, cordials, and other rectified spirits. Beet sugar is preferred in this connection because, as a rule, it has a lower content of dextrans or polysaccharide contaminations which precipitate in the presence of alcohol. Cane sugar, ordinarily higher in dextran content,[13] tends to result in the development of slight turbidity when mixed with alcoholic spirits. Incidentally, beet sugar in the presence of alcohol seems to be particularly effective in producing the symptoms of beet sensitivity.

Several beet-sensitive patients have been observed to develop anthocyaninuria (vegetable pigments in urine) following the ingestion of beets. This confirms Zindler's[14] original observations of the frequency of beet pigments in the urine when beets are ingested by specifically sensitive allergic individuals.

Any of the various types of allergic manifestations may result from beet sensitivity; these include not only asthma, rhinitis, urticaria, atopic dermatitis, headache, and gastrointestinal symptoms but also allergic myalgia,[15, 16] fatigue,[17, 18] and certain mental symptoms of allergic origin.[19] The latter manifestations were illustrated in several of the case histories of this presentation. For reasons not yet apparent, refined sugars of beet or cane origin seem to be particularly effective in producing these constitutional symptoms of allergic etiology in specifically sensitized individuals.

SUMMARY

Clinical evidence bearing on the significance of beet sensitivity is presented. Specific symptoms in beet-sensitive individuals not only develop from the ingestion of beet as a vegetable but also occur from eating granulated beet sugar and large experimental doses of monosodium glutamate of beet origin prepared from the waste of sugar beet processing mills.

The widespread use of products derived from beets, particularly beet sugar, and inexact labeling regulations pertaining to commercially processed foods, alcoholic beverages, and pharmaceutical preparations makes the specific avoidance of beets a difficult task for the individual who happens to be specifically sensitive to beets.

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Physiology

EFFECT OF MONOSODIUM GLUTAMATE ON THE ENDOCRINE AXIS IN RATS.

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Monosodium glutamate (MSG) was administered to neonatal rats in an effort to determine its effects on the endocrine axis. MSG was given in an increasing dose (2.2-4.2/gm bd wt) subcutaneously over 10 days and the rats were autopsied on the 40th day. This treatment resulted in significant decreases in body weight and length in both male and female rats as compared with controls. Absolute and relative weight of the anterior pituitary in male and female MSH treated rats were significantly reduced (Male 4.88 $\pm$ .15 vs 2.40 $\pm$ .13mg, p = .001, Female 4.59 $\pm$ .23 vs 2.02 $\pm$ .09mg, p = .001). Ovaries or testes of MSG treated rats were atrophied with a 68% and 65% decrease in weight respectively. Absolute weight of adrenals and thyroids of MSG treated male and female rats were significantly reduced (Adrenals: Male 34.7 $\pm$ 1.6 vs 24.4 $\pm$ .78mg, p = .001; Female 33.1 $\pm$ 1.3 vs 17.7 $\pm$ .92mg, p = .001; Thyroid: Male 7.99 $\pm$ .31 vs 5.92 $\pm$ .23mg, p = .001; Female 6.98 $\pm$ .3 vs 4.39 $\pm$ .19mg, p = .001). Anterior pituitary content of TSH, LH, FSH, and GH was also determined by bioassay or radioimmunoassay. Pituitary levels of TSH and other trophic hormones were found to be depressed. In conclusion, in rats MSG treatment resulted in marked inhibition of endocrine functions possibly through an effect on the hypothalamus and/or higher CNS center. (Supported by USPHS Grant AM 07467.).

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Effect of Monosodium Glutamate on Some Endocrine Functions[1]

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Abstract

Neonatal female and male rats of the Sprague-Dawley strain were injected subcutaneously with a daily dose of MSG (2.2–4.2 mg/g body wt.) beginning on the 2nd day of life. The rats were autopsied at 40 and 110 days of age. At 40 days of age, the body weight and nasoanal lengths were significantly reduced in the MSG-treated rats. At 110 days, the body lengths of MSG-treated rats were approximately 10–12% shorter than those of control rats, and calculation of the 'Lee' index indicated a significant increase in carcass fat. Food consumption studies, carried out at 75 days of age, showed a significant hypophagia in some MSG-treated rats. Upon autopsy at 40 days of age, the absolute weights of the thyroid and adrenal glands of both sexes were significantly decreased from those of control rats; but when corrected for body weight, these differences were marginal. At 110 days, the absolute weights of the adrenal and thyroid glands of both sexes were significantly reduced from their respective control levels. Gonadal weights of MSG-treated rats were significantly reduced at 40 and 110 days of age. Size and weight of the anterior pituitary glands of both male and female MSG-treated rats were significantly reduced from control values. There was a marked decrease in growth hormone and luteinizing hormone content in the anterior pituitaries of male and female MSG-treated rats at 40 days. However, thyrotropin content of the anterior pituitary of MSG-treated male rats did not show any significant change from control levels at 40 days of age.

Key words
Monosodium glutamate
Body weight decrease
Carcass fat increase
Endocrine gland atrophy
Pituitary hormone content decrease

Several reports have suggested that monosodium glutamate (MSG) may exert a toxic effect when given to both experimental animals and man. The addition of MSG to food has been implicated in the cause of the 'Chinese restaurant' syndrome in man [Schaumburg *et al.*,

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[illegible] studies showed that pharmacological doses of MSG [illegible] to humans precipitated headache, burning sensations, facial [illegible], and chest pains [Schaumburg *et al.*, 1969]. MSG injected [illegible]aneously into neonatal mice has been shown to damage the development of the central nervous system and cause failure of the formation of the inner nuclear layer of the retina [Potts *et al.*, 1960; Olney, 1969]. Olney has also reported that, in mice, MSG selectively destroys the preoptic and arcuate nuclei of the hypothalamus [Olney, 19[illegible]]. It has also been reported that subcutaneous administration of MSG to an infant Rhesus monkey resulted in acute degeneration of hypothalamic neurons [Olney and Sharpe, 1969].

It is well-documented that the hypothalamus secretes specific neurohormones, which are carried to the anterior pituitary gland via the hypophyseal portal vessels, where they exert stimulatory or inhibitory influences on the synthesis and release of the trophic hormones [Schally *et al.*, 1968, 1970]. Chemical or physical damage to the hypothalamus may thus result indirectly in changes in the content and release of the anterior pituitary hormones and, consequently, in changes in the weight of the respective target endocrine organs.

The objective of this study was to determine the effect of administration of MSG on the weights of the anterior pituitary and [illegible] endocrine glands, and on body weight, food consumption, [illegible] length, and obesity. In addition, the anterior pituitary levels [illegible] hormone (GH), luteinizing hormone (LH), and thyrotropin ([illegible]) were measured.

Materials and Methods

[illegible] male rats of the Sprague Dawley strain were injected sub- [illegible] dose of MSG, beginning on the 2nd day of life. The amount [illegible] from 2.2 mg/g body weight on the 1st day until the [illegible] 4.2 mg/g body weight was given, as prescribed by Potts *et al.* [illegible] injections of saline, the volume injected being adjusted to [illegible]. Litters of rats were [illegible] on the basis of sex [illegible], they were separated from their mothers and grouped [illegible] [illegible] with [illegible] [illegible] and weighed on a torsion balance [illegible]

Body weights and nasoanal length were also recorded. The anterior pituitary glands were homogenized in egg albumin-phosphate buffer and aliquots taken for the determination of GH, LH, and TSH content.

TSH was measured by the mouse assay of McKenzie [1958] and the results are expressed in terms of milliunits of NIH-TSH-S-5. LH was measured by the double antibody radioimmunoassay of Niswender *et al.* [1968] (00-RAT-RIA), using a cross-reaction with ovine LH. The amount of LH was expressed in terms of NIH-LH-S-14, which was obtained by direct reading on the standard curve. GH was measured by the double antibody radioimmunoassay of Schalch and Reichlin [1966] and expressed in terms of NIAMD-RAT-GH-R-1. Statistical analyses were performed by the Student 't' test [Snedecor, 1957].

Results

Table IA shows the changes in weight, nasoanal length, and the Lee index in control and MSG-treated male and female rats at 40 days of age. Body weight and nasoanal lengths were significantly reduced

Table IA. Changes in body weight, length, and obesity index in MSG-treated rats at 40 days of age

	Treatment	Body weight (g±S.E.)[a]	Nasoanal length (cm±S.E.)	Lee index[b] (±S.E.)
Males (12)[c]	Controls	161±0.51	24.5±0.21	0.301±0.15
	MSG-treated	104±2.5	21.2±0.45	0.2[illegible]3±0.14
	p[d]	0.001	0.001	NS
Females (12)	Controls	135±3.9	16.9±0.15	0.302±0.15
	MSG-treated	83.2±2.0	14.7±0.12	0.2[illegible]5±0.11
	p	0.001	0.001	NS

Table IB. Changes in body weight, length, and obesity index in MSG-treated rats at 110 days of age

Males (6)	Controls	338±16.0	23.9±0.15	[illegible]
	MSG-treated	302±6.9	21.[illegible]±0.23	[illegible]
	p	NS	0.001	[illegible]
Females (6)	Controls	240±3.7	20.8±0.12	0.2[illegible]
	MSG-treated	[illegible]±13.0	13.4±0.40	[illegible]
	p	NS	0.001	[illegible]

[a] [illegible]
[b] [illegible]
[c] [illegible]
[d] [illegible] Student's 't' test.

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[illegible] Food intake of MSG-treated rats over a 6-day period from 75–81 days of age

[illegible]	Food intake in g ± S.E.[a] 1	2	3	4	5	6
[illegible]						
C[illegible]	26.5±3	22.0±0.8	23.0±1.50	21.5 ±1.04	22.7±1.20	24.5±0.29
[illegible]treated (6)	24.5±2	19.0±1.8	18.7±0.75	18.75±0.25	17.0±0.82	20.0±1.00
p[c]	NS	NS	0.025	0.025	0.01	0.001
[illegible]						
[illegible]s	17.5±0.8	16.0±0.71	17.0±0.71	17.5 ±0.96	18.7±1.50	20.0±0.91
[illegible]	14.7±1.3	13.7±0.85	14.2±1.11	13.7 ±0.85	14.7±0.25	17.0±0.41
p	NS	NS	NS	0.025	0.05	0.025

[illegible] error of mean.
[illegible] in group.
[illegible]d by Student's 't' test.

[illegible] Calculation of the Lee index, a measure of [illegible] the body weight and nasoanal length did not indicate [illegible] fat, and inspection of the body cavity at [illegible] significant accumulation of fat in the body [illegible] in table IB, body weights of the MSG-treated [illegible] 110 days, but were not significantly different [illegible]. However, body length was approximately [illegible] of control rats and calculation of the Lee [illegible] in excess fat. Inspection of the [illegible] of fat in all [illegible], shown in table II, over [illegible] of age, did not [illegible] 110 days [illegible] [illegible] for body weight, [illegible]

of [illegible] normal [illegible]

4 [illegible] normal.

Table III also shows that the absolute weights of the adrenal glands of MSG-treated rats at 40 days of age were significantly decreased, but not when corrected for body weight. However, at 110 days the relative and absolute adrenals weights were significantly reduced from control levels.

Gonadal weights of the MSG-treated rats were significantly reduced at 40 and 110 days of age as shown in table IV. The testes of MSG-treated rats appeared grossly normal, but with an over-all reduction in mass. Although the absolute weights of the testes at 40 and 110 days were significantly decreased, when corrected for body weight at 110 days, the differences were not significant. In females treated with MSG, the ovaries appeared atrophied, with many atretic follicles.

Table III. Thyroid and adrenal weights of control and MSG-treated rats at 40 and 110 days

	40 days (12)[b]		110 days (6)	
	Thyroid wt. mg ± S.E.[a]	Thyroid wt./100 g body wt. ± S.E.	Thyroid wt. mg ± S.E. (6)	Thyroid wt./100 g body wt. ± S.E.
Males				
Controls	7.99±0.31	5.96±0.13	10.70±0.63	3.01±[illegible]
MSG-treated	5.92±0.23	5.64±0.16	8.78±0.33	2.7[illegible]
p[c]	0.001	NS	0.025	NS
Females				
Controls	6.98±0.30	5.19±0.21	10.15±0.70	4.21[illegible]
MSG-treated	4.30±0.19	5.31±0.26	7.96±0.55	3.5[illegible]
p	0.001	NS	0.05	NS
	Adrenal wt. mg ± S.E.[a]	Adrenal wt./100 g body wt. ± S.E.	Adrenal wt. mg ± S.E.	Adrenal wt./100 g body wt. ± S.E.
Males				
Controls	[illegible]±1.6	21.6[illegible]	41.1[illegible]	1[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
p	[illegible]	NS	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	71.2[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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There was an approximate 68% decrease in ovarian weight at 40 days and [illegible]% at 110 days of age, and these differences persisted when corrected for body weight.

Table IV. Gonadal weights of control and MSG-treated rats at 40 and 110 days

	40 days (12)[b]		110 days (6)	
	Testes g ± S.E.[a]	Testes wt./100 g body wt. ± S.E.	Testes g ± S.E.	Testes wt./100 g body wt. ± S.E.
Males				
Controls	1.90 ± 0.07	1.17 ± 0.02	3.27 ± 0.11	0.93 ± 0.09
MSG-treated	0.66 ± 0.08	0.62 ± 0.08	2.60 ± 0.09	0.8 ± 0.03
p[c]	0.001	0.001	0.005	NS
	Ovaries mg ± S.E.	Ovaries wt./100 g body wt. ± S.E.	Ovaries mg ± S.E.	Ovaries wt./100 g body wt. ± S.E.
Females				
Controls	38.6 ± 2.9	28.6 ± 1.92	76.7 ± 2.8	31.9 ± 1.0
MSG-treated	12.3 ± 2.8	14.8 ± 0.92	39.7 ± 6.3	17.2 ± 2.6
p	0.001	0.001	0.001	0.001

[a] ± Standard error of mean.
[b] Number of animals in group.
[c] Probability as determined by Student's 't' test.

Table V. Anterior pituitary weights in control and MSG-treated rats at 40 and 110 days

	40 days (12)[b]		110 days (6)	
	Ant. pit. mg ± S.E.[a]	Ant. pit. wt./100 g body wt. ± S.E.	Ant. pit. mg ± S.E.	Ant. pit. wt./100 g body wt. ± S.E.
[illegible]	[illegible] ± 0.15	3.03 ± 0.05	7.20 ± 0.36	2.02 ± 0.085
[illegible]	[illegible] ± 0.13	2.27 ± 0.08	4.32 ± 0.29	1.34 ± 0.090
[illegible]	0.001	0.001	0.001	0.001
[illegible]	[illegible]	3.41 ± 0.15	10.11 ± 0.57	4.20 ± 0.20
[illegible]	[illegible]	[illegible] ± 0.12	3.80 ± 0.67	[illegible] ± 0.31
[illegible]	[illegible]	[illegible]	0.001	0.001

[a] ± Standard error of mean.
[b] Number of animals in group.
[c] Probability as determined by Student's 't' test.

Table V shows the weights of the anterior pituitaries from MSG-treated and control rats. The size and weight of the anterior pituitary (glands) of both male and female MSG-treated rats were significantly reduced from control values. There was a more than 50% reduction in weight of the anterior pituitary glands in both male and female MSG-treated rats at 40 days of age, and this decrease in weight persisted throughout the remainder of the experiments.

It is of interest to determine whether the changes in weight of the anterior pituitary gland and target glands might be associated with changes in content of trophic hormones of the anterior pituitary. Table VI show the levels of GH and LH in the anterior pituitaries of control and MSG-treated male and female rats. There was a marked decrease in GH (71–84%) content in anterior pituitaries of male and female MSG-treated rats. Total content of LH was also markedly reduced in male and female MSG-treated rats. TSH content of the anterior pituitaries of MSG-treated male rats failed to show any significant change from controls when measured at 40 days of age.

Discussion

Many of the effects produced by MSG in neonatal rats are similar to a syndrome seen in weanling rats with electrolytic lesions in the ventromedial nucleus (VMN). This syndrome, as described by FROH-

Table VI. Anterior pituitary content of GH, LH, and TSH in control and MSG-treated rats at 40 days

	μg GH total anterior pit. ± S.E.[a]	mμg LH total anterior pit. ± S.E.	mU TSH total anterior pit. ± S.E.
Males (3)[b]			
Controls	288.5 ± 3.4	6647 ± 393	60[illegible] ± 95
MSG-treated	82.6 ± 29.6	1742 ± 173	[illegible] ± 49
p[c]	0.001	0.001	NS
Females (3)			
Controls	318.2 ± 12.2	3121 ± 285	
MSG-treated	51.0 ± 11.3	1353 ± 42	
p	0.001	0.005	

[a] ± Standard error of mean.
[b] Number of animals in group.
[c] Probability as determined by Student's 't' test.

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[illegible] [illegible], consists of, among other changes, a decrease in [illegible] with decrease in pituitary and plasma GH levels, and [illegible] in carcass fat unassociated with an increase in total body [illegible] or in food intake. In our studies, MSG treatment resulted in [illegible] approximately 10–15% decrease in the nasoanal length at 40 and [illegible] of age. While upon inspection the MSG-treated rats appear[illegible] at 110 days, there was no overt obesity in terms of increased [illegible] weight over that of the controls.

A rapid means of determining obesity, without sacrificing the [illegible], consists of dividing the cube root of the body weight by the [illegible] length. The resulting figure is referred to as the 'Lee' or [illegible]'y' index, and it is a measure of obesity [Bernardis and Skel[illegible], 1967]. Recently, Bernardis and Patterson [1968] have shown [illegible] correlation between the Lee index and body fat in rats with [illegible] lesions in the VMN. When our data were calculated [illegible] formula, there was a significant increase in the Lee index [illegible] at 110 days, even though the weights of MSG-treated [illegible] not exceed the body weights of control rats. There was a [illegible] increase in fat deposit both in the subcutaneous and body [illegible]. Food consumption measured at 75 to 81 days of age [illegible] that hyperphagia was the cause of this obesity. Food [illegible] indicate occasionally significant hypophagia in [illegible] rats. As in weanling rats made obese by VMN [illegible] may result from a metabolic and/or hormonal [illegible] of weanling VMN-lesioned rats, Frohman *et al.* [illegible] an increase in plasma triglycerides to a value [illegible] controls. When plasma triglycerides were mea[illegible] at 150 days of age, there was an indica[illegible] in most rats. However, the results were [illegible]

[illegible] in MSG-treated rats possibly resulted from [illegible] synthesis by the anterior pituitary [illegible] marked decrease in pituitary GH [illegible]. There is now good evidence that the [illegible] GH is controlled by GH-[illegible] [illegible]

electrolytic lesions is also associated with growth retardation [Bernardis *et al.*, 1963; Han *et al.*, 1965; Krulich *et al.*, 1965; Bernardis and Skelton, 1966]. Hypothyroid rats similarly show a decreased pituitary GH content [Knigge, 1958]. It is unlikely that these MSG-treated rats were hypothyroid, since the TSH content of the anterior pituitary was normal and the weight of the thyroid and its uptake of labelled iodine was not affected at 40 days of age. Thyrotropin-releasing hormone (TRH), which controls the release and synthesis of TSH, may be produced in a rather diffuse portion of the anterior hypothalamus and may have escaped damage in the MSG-treated rats [D'Angelo, 1963].

In contrast, both ovaries and testes of MSG-treated rats were greatly affected. At 40 days, there was a 68% decrease in the weight of the ovaries from MSG-treated rats. This decrease was evident even when corrected for body weight at 40 and 110 days. In males, the testes were normal in appearance, but showed an over-all reduction in mass. Absolute weight of the testes at 110 days was significantly decreased, but not when corrected for body weight. It is not known whether these males or females were fertile. It is interesting to note that pituitary levels of LH were decreased in MSG-treated rats of both sexes, when measured at 40 days. Whether follicle-stimulating hormone (FSH) levels were also reduced, was not determined. The decrease in testicular weight and adrenal weights of MSG-treated rats is in contrast to results reported in mice by Olney [1969]. In mice treated with MSG, the testes were reported to be indistinguishable from those of control mice, and there was a suggestion of mild adrenocortical hypertrophy. Perhaps these discrepancies may be explained by a degree of difference in sensitivity between mice and rats to MSG, i.e., the extent of the hypothalamic lesion in rats, produced by the administration of MSG, may be larger than that produced in mice.

We have not ruled out direct toxic effects of MSG on the [illegible] glands themselves. Similarly, no histological studies were performed in the experiments. In mice, histological changes have been noted in the hypothalamus but not in the anterior pituitary [illegible] [illegible]

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[illegible] of the hypothalamic releasing hormones that control [illegible] and synthesis of the anterior pituitary hormones. Further [illegible] on the content of hypothalamic releasing hormones from [illegible]-treated rats may be needed to provide conclusive evidence for this hypothesis. If this concept is correct, then the MSG-treated rat may prove to be a useful experimental model for studying the effects of lowered release of hypothalamic hormones, and the correction of such effects by replacement therapy.

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Distilled water was administered in an identical manner to five infant monkeys (controls).

Nineteen animals were obtained from dated conceptions delivered in the Primate Breeding Facility of the University of Illinois at the Medical Center. The other two were purchased from a commercial supplier. In two instances, infants were delivered at term by cesarean section. The two species, *Macaca mulatta* and *Macaca irus*, are similar, although *M. irus* is a smaller animal, which is reflected in its lower weight at birth. The conditions of the experiment are shown in Table 1. Infants were fasted for 4 hours before being given, by stomach tube, a 50 percent solution of MSG in distilled water. Control animals received only distilled water by stomach tube. The doses given (1, 2, and 4 g/kg) included levels both higher and lower than that (2.7 g/kg) of Olney and Sharpe (*6*). We chose to administer MSG by stomach tube rather than by injection because its use by man is primarily as a food additive. Each infant was maintained in an incubator with handling and cuddling at intervals for a 6-hour period. No unusual behavior was exhibited by the infants.

At the end of the 6-hour period, the infant monkey was sedated with Sernylan (1 mg/kg), and an endotracheal tube was inserted, either through the oral pharynx or a tracheostomy incision. Halothane was administered by positive pressure (2 percent), and a thoracoabdominal incision was made. The abdominal aorta was clamped, and a 5 percent solution of sodium nitrite was injected into the left ventricle. A cannula was threaded through the left ventricle into the aorta and Tyrode solution containing gum acacia was perfused for 2 minutes. This was followed immediately by a perfusate of 19 percent glutaraldehyde (25 ml). The third perfusate consisted of 2 percent glutaraldehyde with acrolein and gum acacia in phosphate buffer. Solutions were prepared according to Schultz (*7*).

To diminish rapid cerebral vasoconstriction at the time of perfusion, six monkeys were chilled to 26°C (rectal temperature) by placing them on ice bags for approximately 1 hour prior to perfusion. In these instances gum acacia was not added to the perfusing solutions.

At the end of the perfusion, the head was removed and stored over-

Monosodium Glutamate: Absence of Hypothalamic Lesions after Ingestion by Newborn Primates

Abstract. *After receiving monosodium glutamate by stomach tube, the brains of infant macaques were perfused for examination by light and electron microscopy. No morphological differences were observed in the hypothalamic regions of treated and control monkeys. However, inadequately fixed tissue had the same appearance as that of the previously reported brain lesion in a newborn monkey.*

The nervous system in certain neonatal species appears to be highly susceptible to injury after the injection or oral administration of large doses of monosodium glutamate (MSG). Acute neuronal necrosis has been reported to occur in the retina of neonatal mice (*1, 2*) with concomitant biochemical (*3*) and electrophysiological (*4*) alterations. It has been reported that the subcutaneous injection of MSG (0.5 to 4.0 g per kilogram of body weight) in the newborn mouse is followed by a lesion in the arcuate nucleus (*5*). Further, newborn mice receiving daily subcutaneous injections of MSG over a 10-day period developed into obese adults with delayed skeletal maturation. Female mice so treated were reported to be sterile (*5*). Because of its serious implications for the human infant whose diet may contain MSG, the most disquieting report has been that of a hypothalamic lesion in the periventricular-arcuate nucleus 3 hours after the injection of MSG into a single newborn rhesus monkey (*6*).

We administered MSG by nasogastric tube to 16 infant monkeys and then examined the hypothalamic region by both light and electron microscopy.

Fig. 1. (A) Paraffin section through arcuate nucleus–median eminence region from neonate monkey which had received 4 g of MSG per kilogram of body weight (× 90). (B) Epon section of ventral hypothalamus cutting through infundibular stalk from another monkey receiving MSG (4 g/kg). Sections such as these were indistinguishable from comparable regions of control monkeys (× 90).

night in the final perfusate. The following day, two or three sample blocks (1 mm thick slices) were cut through the arcuate–median eminence area with a Sorvall TC-2 tissue sectioner. These slices were post-fixed in osmium, dehydrated, and embedded in Epon. Sections (1 μm thick) were cut and stained with methylene blue–azur II and examined with the light microscope. Ultrathin sections of each block were then examined with an RCA EMU 3H microscope.

For light microscopic examinations, monkeys were perfused with physiologic saline followed by 10 percent formalin containing gum acacia. Serial paraffin sections of the hypothalamus extending from the preoptic area to the mammillary bodies were cut at 15 μm and stained with cresyl violet.

The hypothalamic area was prepared for light microscopic examinations in eight infant macaques and for electron microscopic examinations in 13. The hypothalamus was first studied by light microscopy in order to evaluate the presence or absence of a lesion induced by MSG, because light microscopy has been shown by Olney to be adequate for detecting retinal (*2*) and hypothalamic (*5*) lesions. Also, at the light microscopic level, we have been able to confirm hypothalamic lesions in the mouse (*8*) characterized by swollen cell somas and pyknotic nuclei. Close examination of serial paraffin sections of the hypothalamic areas derived from infant monkeys that were treated with MSG revealed no differences between these sections and comparable serial sections obtained from control animals (Fig. 1A). Similarly, examination of 1 μm Epon sections prepared from 16 sample blocks of the arcuate–median eminence area revealed no significant differences between control and treated animals (Fig. 1B). Dendrites and cell bodies of neurons were normal in appearance at all dosage levels.

Ultrathin sections of the arcuate nucleus confirmed our light microscopic observations in that no significant differences were found in the appearance of the cell somas and their nuclei between normal infant monkeys and those treated with MSG (Fig. 2, A and B). However, electron microscopy did reveal certain details not readily apparent in paraffin and Epon sections. Badly to marginally fixed areas became readily observable and in these regions were seen swollen dendrites lacking internal cytoplasmic contents (Fig. 3) as well as neuronal perikarya exhibiting a spectrum of "degenerative" changes. As is often true of inadequately perfused brain, we found that poor fixation characterized one brain region while immediately adjacent areas presented a well-fixed appearance.

No significant morphological difference at the light or ultrastructural level could be detected in the periventricular-arcuate area between neonatal monkeys which served as controls and those ingesting MSG. In small areas

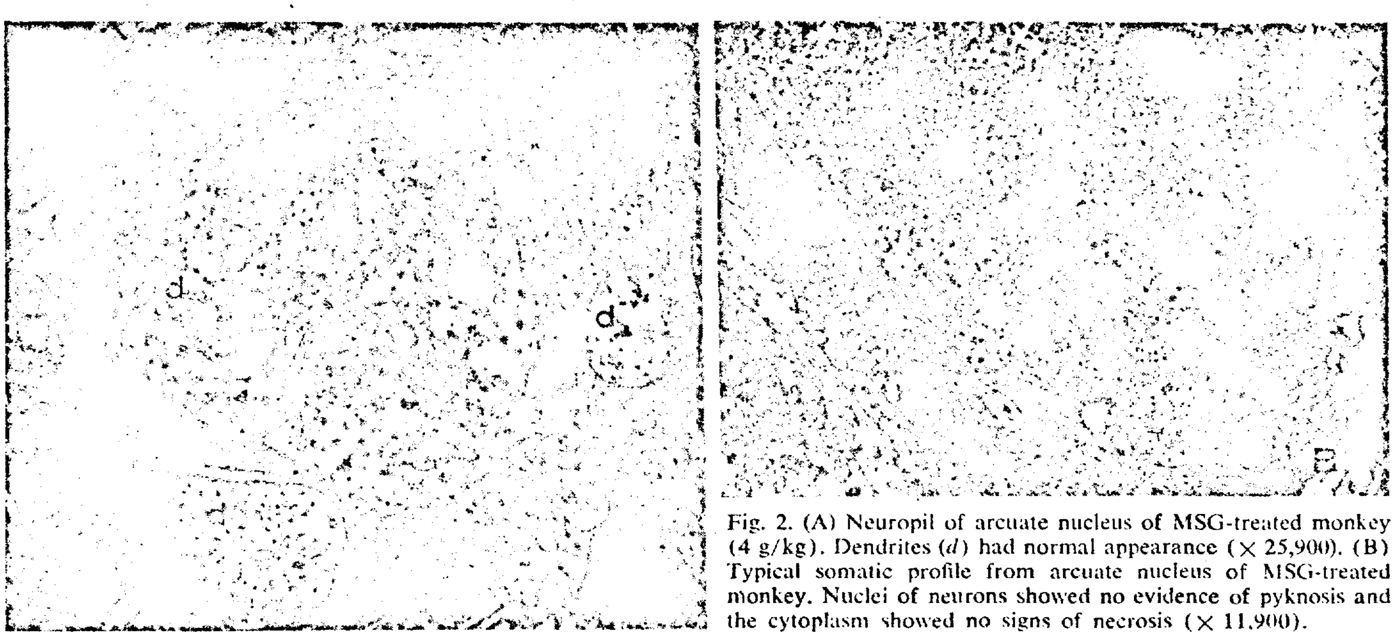

Fig. 2. (A) Neuropil of arcuate nucleus of MSG-treated monkey (4 g/kg). Dendrites (*d*) had normal appearance (× 25,900). (B) Typical somatic profile from arcuate nucleus of MSG-treated monkey. Nuclei of neurons showed no evidence of pyknosis and the cytoplasm showed no signs of necrosis (× 11,900).

Table 1. Species, age, body weight, and administered dose of MSG.

Species	Age (days)	Weight (g)	MSG dose (g/kg)
Electron microscopic examination			
M. irus	9	310	Water
M. irus	21	360	Water
M. irus	36	335	Water
M. mulatta	0.02	550	1
M. mulatta	2	450	1
M. mulatta	4	430	1
M. irus	7	445	2
M. mulatta	8	390	2
M. irus	3	320	3
M. irus	2	345	4
M. irus	3	340	4
M. irus	6	300	4
M. irus	8	300	4
Light microscopic examination			
M. irus	15	310	Water
M. irus	51	410	Water
M. irus	1	510	2
M. irus	4	375	2
M. irus	6	320	2
M. mulatta	8	240	2
M. irus	8	340	4
M. mulatta	14	480	4

of the periventricular-arcuate region in both normal and treated infants, poorly fixed tissue appeared similar at the ultrastructural level to that described in a newborn monkey after MSG administration (*6*). The obstacles to obtaining consistently superior fixation, especially in a large animal, do create difficulties in interpreting neuronal pathology. For these reasons, it appears imperative that such studies be performed on a number of animals, that wide-scale sampling of both experimental and control blocks be undertaken, and that the electron microscopic appearance of controls be examined with great care.

According to Olney (*2*, *5*), the lesion induced by MSG in the rodent is characterized by swollen dendrites and cell bodies accompanied by mitochondrial transformations. Degeneration leading to neuronal necrosis was followed by completion of phagocytosis 48 hours after injection. Arees and Mayer (*9*) have described necrotic areas in the arcuate nucleus of the mouse resulting from degenerating microglia a few hours after MSG treatment. We, too, have been able to confirm reproducibly a lesion in the arcuate nucleus of the newborn mouse and in other brain areas in response to the ingestion of MSG (*8*).

With respect to both functional and morphological indices of maturation, the central nervous system of the newborn primate and the newborn rodent are hardly comparable. It remains to be determined whether it is glutamate or one of its metabolites that is responsible for the damage observed in the newborn mouse. Conceivably, slight species differences in the metabolism of glutamate may make the rodent mouse more susceptible to neuronal damage than the primate. The final parameter that may vary with developmental age or on a species basis is that of route of access to the area susceptible to injury. The blood-brain barrier, the cerebrospinal fluid, and the integrity of the ependyma lining the ventricles all deserve closer scrutiny in this respect.

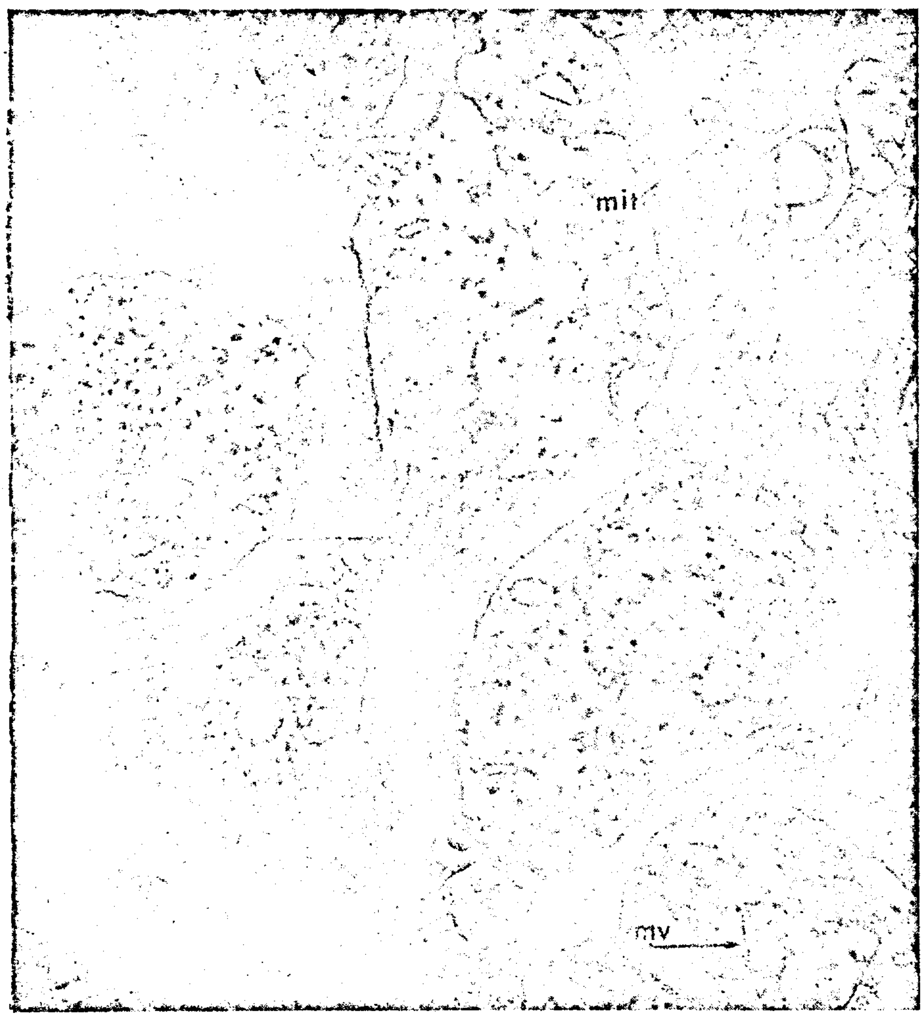

Fig. 3. Portion of neuropil from arcuate nucleus of control monkey in which quality of fixation was poor. In such areas ballooning of dendrites, as shown at arrow, was relatively frequent. Also, membrane vesiculation (*mv*), mitochondrial swelling (*mit*), and vacuolization of organelles in neuronal profiles was often found. Epon sections, 1 μm, which preceded ultrathin sections, showed a "Swiss-cheese effect" because of dilation of badly preserved dendritic processes ($\times$ 17,000).

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SIN CIB-SYN: ACCENT ON GLUTAMATE

To the Editor: To suppress the mounting hysteria and prevent the wholesale slaughter of Chinese-restaurant owners, we feel impelled to present a preliminary communication on the etiology, psychopathology and clinical pharmacology of the variously misnamed post-sino-cibal syndrome (Chinese-restaurant syndrome). This reaction although first described in the technical literature in your columns has been, in fact, well known for many years to experienced allergists and Chinese-restaurant owners.

The reaction is brought on in susceptible subjects by monosodium glutamate (supplied to Chinese restaurants as Accent, as Ajinomoto powder or as monosodium glutamate, C.P.). Five gm of material is adequate to produce a response. In nonsusceptible persons 25 gm will not produce the reaction.

With the enthusiastic co-operation of the Shanghai Cafe one of us ate Chinese food for breakfast, lunch and dinner until the search had been narrowed to either hot and sour soup or wonton soup, both of which produced the reaction. A rough filtrate of wonton soup also produced the reaction. Upon sampling of the individual ingredients, the dagger of suspicion pointed at monosodium glutamate. Further experiments confirmed this suspicion. The experiments were performed with the use of approved blind and double-blind technics on three volunteers. If the suspicion that irresponsible human experimentation was done has crossed your mind, be at ease. The days of Walter Reed are not past.

The reaction is characterized by an onset time of 15 to 25 minutes, a duration of 45 minutes and a fluctuating intensity. The subject first feels a burning sensation in the back of the neck, followed by a burning over the forearms and anterior thorax. This is followed by a feeling of infraorbital pressure and tightness. There is a variable amount of substernal discomfort. The burning is without erythema or pruritus, and the tightness is without visible signs of muscular contraction. Many previously described components of this reaction — that is, syncope, tachycardia, lacrimation, fasciculation and nausea — can be attributed to anxiety and fear of face loss (pseudopostsinocibaldefaciation). A group response sometimes clouds the pure reaction. This is not dissimilar from the reaction described in Polynesian cultures called *Latah (M. J. Indian Archipelago*, 1875).

The reaction is not affected by pretreatment with diphenhydramine and cannot be simulated by ingestion of equivalent amounts of sodium in the form of sodium chloride or by glutamic acid (l-form). As many as eight episodes a day can be survived (personal experience).

This letter does not pretend to be the last word. Experiments to determine whether it is monosodium glutamate or a contaminant are in progress. Despite cuts in the budget of the N.I.H., basement research will still give the answers.

Our thanks are due first to the untold number of victims who have called in the middle of the night, because this above all has driven us forward to find an answer. However, we must also thank the swelling group of colleagues who have contributed their efforts, notably Dr. Jack Peisach, Dr. Peter Engel and Mr. Brooke Jennings.

Proceeds from the soon to be written monograph, *The Race for the Double Wonton*, will be used to improve working conditions for the dedicated investigators.

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Monosodium L-Glutamate: Its Pharmacology and Role in the Chinese Restaurant Syndrome

Abstract. *Monosodium L-glutamate is the cause of the Chinese restaurant syndrome and can precipitate headaches. In appropriate doses it causes burning sensations, facial pressure, and chest pain. These are pharmacological effects obeying a dose-effect relationship. There is considerable variation in oral threshold doses among individuals.*

Monosodium L-glutamate (MSG) is a widely used food additive. Twenty thousand tons of MSG are manufactured and used in the United States each year (*1*). The labeling of a widely used brand states, "To wake up all the flavor nature put in your food, be sure to use at least the amounts . . . suggested below, adding more as desired." Amounts approximating 1 g per serving are the minimum amounts suggested.

Monosodium L-glutamate is not a wholly innocuous substance. It was proposed as the cause of the Chinese restaurant syndrome in July 1968 (*2*). We report here some aspects of the acute human pharmacology of MSG and, in addition, present evidence that it causes headache.

Many symptoms have been suggested as components of the syndrome (*3*). On repeated observations, we find that three categories of symptoms can be elicited by MSG—burning, facial pressure, and chest pain. Headache is a consistent complaint in a minority of individuals. The MSG response and the syndrome are identical. The symptoms appear only if the meal is taken on an empty stomach by a susceptible individual (*4*).

The proof that MSG is the cause of the syndrome was arrived at with the cooperation of two subjects, both of whom had symptoms in the same restaurant. We found that 200 ml of wonton soup alone was sufficient to provoke an attack (*5, 6*). Although other foods caused the response, wonton soup was the simplest in composition.

The restaurant prepared soup without MSG and it failed to provoke an attack. The subjects then ingested each of the seven components of the wonton soup separately. Only MSG caused the symptoms. In a blind procedure, MSG was then given to four additional individuals who had symptoms in the same restaurant. It provoked an attack in all four in amounts of 3 g or less. Therefore, we concluded

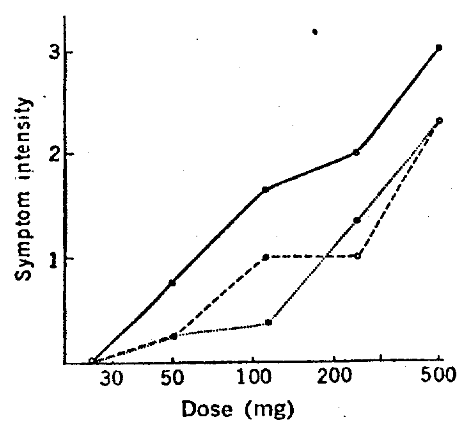

Fig. 1. Relation between intensity of burning (solid line), facial pressure (dashes), and chest pressure (dotted line) and intravenous dose of MSG. Each point represents a mean intensity from three or more responses. The data were obtained from four subjects.

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that the Chinese restaurant syndrome was caused by MSG.

We then determined that L-glutamic acid in several forms could provoke an attack. Symptoms were provoked in our two original subjects by Accent (3 g), MSG, chemically pure grade (C.P.) (3 g), monopotassium L-glutamate C.P. (4 g), DL-glutamic acid (5 g), and L-glutamic acid (5 g). A previous report of failure of L-glutamic acid to provoke an attack (2) was due to our using an insufficient volume of water as a vehicle for this poorly soluble substance.

A repeat trial with 5 g of L-glutamic acid, fully dissolved in 500 ml of water at 30°C, provoked an attack. In addition, to eliminate the possibility of an impurity in the commercially available L-glutamate, we synthesized monosodium DL-glutamate (7). The resultant product was identified from infrared spectra and by thin-layer chromatography. Five grams of this product were sufficient to provoke an attack.

The following substances did not provoke symptoms: monosodium D-glutamate (7 g), monosodium L-aspartate (5 g), NaCl (10 g), and glycine (5 g).

We next determined that the intensity and duration of the symptoms were related to the dosage of MSG. To define the temporal sequence and nature of the symptoms, we gave MSG as Glutavene, intravenously to 13 subjects. After oral administration of this substance, the symptoms were perceived by our subjects in a less well-defined order because the onset was less abrupt and the increase in intensity more subtle. After oral administration many subjects experienced only one or two components of the syndrome. Fifty-six normal subjects (30 male and 26 female) were given oral MSG. The age range was from 21 to 67 years. Symptoms of the syndrome occurred in all but one subject. We gave MSG to 36 subjects at different doses to determine the distribution of thresholds. In the one individual in whom no symptoms could be produced despite large doses of oral MSG (21 g), symptoms were produced with an intravenous dose of 50 mg.

We previously reported that one of the subjects had ingested 25 g of MSG without symptoms (2). However, on repeated testing this individual showed a threshold of 5 g. The symptoms had been obscured, in the previous test, by prostration and gastric distress which was provoked by the 25-g dose.

After intravenous administration in 13 subjects, the first symptom appeared in 17 to 20 seconds. The threshold range (for minimum symptoms) was 25 to 125 mg. After intravenous injection of a suprathreshold dose, the first symptom to appear was a burning sensation, usually beginning over the chest and spreading subsequently to the neck, shoulders, forearms, the abdomen, and occasionally the thighs. This varied in intensity from a "mild sunburn" sensation with threshold dosage to a severe "scorching" sensation with large dosage. We refer to this as the "burning sensation" in our data and graded its intensity on a three-point scale, depending on whether it was just perceptible (1 point), moderately uncomfortable (2 points), or severe (3 points).

The second category of symptoms was a sensation of tightness and pressure over the malar areas, occasionally extending into the zygomatic and retro-orbital regions. This was sometimes described as similar to postanesthetic numbness. This sensation was usually the last to appear and had the longest duration. After intravenous injection it lasted 120 to 180 seconds; after oral administration it lasted for 30 minutes. The intensity of this "facial pressure" was also graded on a three-point subjective scale.

The third type of symptom was a sensation of pressure over the precordium or substernal area, occasionally radiating to the axillae or neck. This sensation, referred to as the "chest pressure," followed the burning by about 5 seconds. Its intensity was graded in the same manner. This sensation can be very alarming; in the past, one of our susceptible physician subjects requested an electrocardiogram after a Chinese meal. The subjects who received 500-mg infusions of MSG showed no electrocardiographic changes despite the presence of severe chest pain.

Dose-effect curves for intravenous MSG are shown in Fig. 1. Oral administration of MSG caused symptoms after 15 to 25 minutes. The oral threshold range for minimum symptoms in 36 subjects was from 1.5 to 12 g. The distribution can be seen in Fig. 2. The oral threshold bore no relationship to the intravenous threshold in the same person. There was no apparent relation of threshold to body weight, sex, or age.

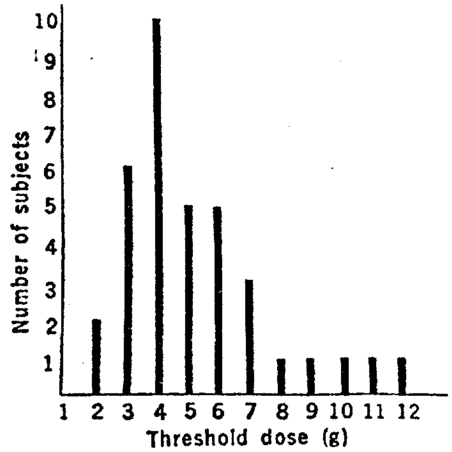

Fig. 2. Oral thresholds for minimum symptoms of MSG response in 36 subjects.

The oral dose-effect curves (Fig. 3) also show an increase in intensity of response with increase in dosage. Single-blind methods were used for the dose-response experiments. In two subjects, the ingestion of 50 mg of diphenhydramine and 600 mg of aspirin on separate occasions did not affect the response to oral MSG.

In two subjects, antecubital intravenous injection of MSG, with the arterial circulation occluded by an axillary cuff [a method of regional arteriovenous perfusion (*8*)] produced burning of the subject's entire arm. Seventeen seconds after we removed the cuff, releasing the substance into the rest of the body, the subjects felt the burning sensation over the chest and neck. The burning sensation is therefore a peripheral phenomenon and not due to central nervous system stimulation.

Six subjects complained of headache after oral ingestion of MSG. Review of the medical history of all our subjects showed six with a history of common migraine and three with a history of combined vascular-muscle tension headache (*9*). Those six who had originally complained of headache after MSG came from this group of nine. A double-blind placebo-controlled experiment on eight individuals with headache history, including five of the previous six reactors, revealed two subjects who consistently suffered from headache after MSG.

The double-blind experiment was conducted as follows. Homemade chicken broth was used as a vehicle. Six grams of MSG were added to one of three cups of broth. All solutions were then salted to a close approximation of identical taste. Each day, on awakening, the subjects took a cup of broth and made a written report on all symptoms. The headache, described as pressure and throbbing over the temples and a band-like sensation around the forehead, started 20 to 25 minutes after ingestion of the MSG and lasted 1 hour. No nausea or warning were noted in this double-blind experiment.

The oral dose-effect curves of MSG indicate that the Chinese restaurant syndrome is the normal response to this agent. All subjects tested could be made to experience the sensory phenomena if they ingested enough MSG or if they had an intravenous injection of as little as 125 mg. All of our subjects with the syndrome had oral thresholds of 3.0 g or less.

Because prior ingestion of food, which delays absorption of the agent, will protect even the most susceptible individuals from this phenomenon, it is not surprising that the soups, which are usually eaten first on an empty stomach and contain large amounts of MSG, provoke the symptoms.

It has been suggested that people sensitive to small amounts of MSG are carriers of an inherited trait, as are those who have taste sensitivity to phenylthiourea (*10*). We studied three families with more than one known susceptible individual; no pattern has emerged to indicate a genetic mechanism.

Since commercial MSG is manufactured by a yeast fermentation process, it was imperative to rule out an organic contaminant. Such an impurity recently produced a supply of unpleasant-tasting MSG in Suchow (*11*). The presence of an impurity has been ruled out by

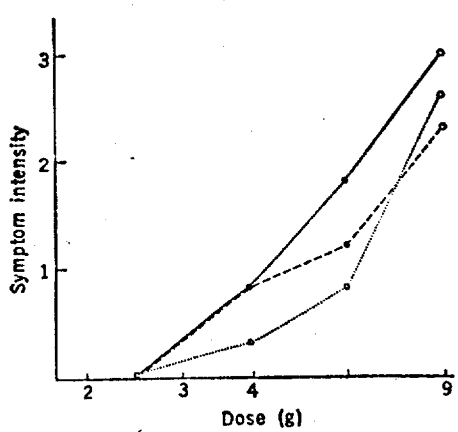

Fig. 3. Relation between intensity (see text) of MSG response and oral dose in grams. Solid line, burning; dashes, facial pressure; and dotted line, chest pressure. Each point represents a mean intensity from four or more responses. The data were obtained from six subjects.

the positive reaction to the synthesized product.

It is difficult to define an exact mechanism for the responses to MSG. Since L-glutamic acid is present in large amounts in the central nervous system and has been suggested as a neurohumoral transmitter (*12*), it seemed reasonable to assume a central nervous mechanism for all the sensory phenomena. However, the results of the local perfusion of one extremity isolated from the rest of the body has dispelled this theory as accounting for all the symptoms. There is usually no sensation immediately after intravenous administration of the substance. However, after 17 seconds there is an intense burning, first in the chest, then spreading centripetally to involve the shoulders, neck, forearms, and abdomen.

In two of the six individuals given 500 mg intravenously, the burning sensation traveled down the midline of the abdomen, bifurcated and continued into the thighs. This response, combined with the isolated limb perfusion, implicates arterial receptor. The chest pressure after intravenous injection seemed somewhat like anginal pain. This pain is also similar to that produced on stimulation of the aortic chemoreceptors (*13*). The lack of change in electrocardiogram would be consistent with either.

Headache occurred consistently in two of our 56 subjects; it was the primary complaint of two correspondents in the original description of the disease; and we have received four letters from individuals in whom headache was the only symptom. The headache pattern is that of a combined vascular-muscular contraction headache. The significance of L-glutamic acid in the epidemiology of this common headache is still undetermined.

A review of experiments on oral and intravenous administration of MSG has revealed none of the reactions reported here. Because in the two conditions for which MSG has been prescribed (hepatic coma and mental retardation) the subjective response of the individual is unreliable if elicitable at all, it is not surprising that the symptoms have not been reported.

Since glutamic acid is present in large amounts both in the body and in some foods, the Food and Drug Administration places MSG in the category termed "generally regarded as safe." No limitation is placed on its use as a food additive (*1*). We now have shown that MSG can produce undesirable effects in the amounts used in the preparation of widely consumed foods.

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BRAIN DAMAGE IN THE MALE DOMESTIC FOWL TREATED WITH MONOSODIUM GLUTAMATE

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A single subcutaneous injection of monosodium glutamate (MSG) in 2 to 9 days old mice induced neuronal necrosis in several brain regions, including the hypothalamus (Olney, 1969). Brain damages in an infant rhesus monkey, rabbits and rats were also induced by a single subcutaneous MSG injection (Olney and Sharpe, 1969). On the other hand, Adamo and Ratner (1970) in a recent publication, reported on the lack of such effects on rats' brains.

The effect of MSG on certain physiological processes was included in our studies of the domestic fowl's neuroendocrine control. This report is concerned with the effect of MSG administrations on the anatomical changes in the central nervous system. Further effects of MSG administration on the chicken are under investigation.

Eighty-four, 5 day old New Hampshire × White Leghorn cross-bred male chicks, were divided into 6 equal groups. All groups were subcutaneously injected in the dorsal part of the neck, according to the

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following order:

	Treatment†		Volume (ml.)
Group 1	1 mg. MSG†/gm. B.W.	single inj.	0.3
Group 2	1 mg. MSG†/gm. B.W.	one daily inj./10 days	0.3
Group 3[1]	4 mg. MSG/gm. B.W.	single inj.	1.2
Group 4[2]	4 mg. MSG/gm. B.W.	one daily inj./8 days	1.2
Group 5	Saline	single inj.	0.3
Group 6	Saline	one daily inj./10 days	1.2

† MSG manufactured by B.D.H. chemical division, England.
[1] Three chicks died 24 h. post injection.
[2] Five chicks died during the treatment.

Forty days after start of injections, 3 birds from each group were killed by decapitation and their brains immediately removed and fixed in 10% neutral formalin. After embedding in 10% gelatin, 15μ. frozen serial frontal sections were performed and stained with thionine. The brains were sectioned in a position similar to the plane described by Van Tienhoven and Juhasz (1962), in the atlas of the chicken brain.

The brain sections of the MSG treated and saline treated control birds were examined by light microscopy. While brain damage was found in each of the MSG treated birds, none was observed in the controls. The typical brain damage was located in

FIG. 1. Brain frontal section through the hypothalamus region of a 45 days old male chicken treated with 1 mg. MSG/gm. B.W./day, from 5 to 15 days of age. The picture depicts the center of the damaged area. The damaged area is on both sides of the brain, near the midline, close to the third ventricle. It extends dorsally to the *subrotundus nuclei*, laterally to the *rotundus nuclei*, and ventrally to the *tr. opticus marginalis*, including the *arcuate nuclei*. The *ventromedial hypothalamic nuclei* (arrow) look unaffected. Note the condensed cells zone surrounding the damaged area. (× 5.1).

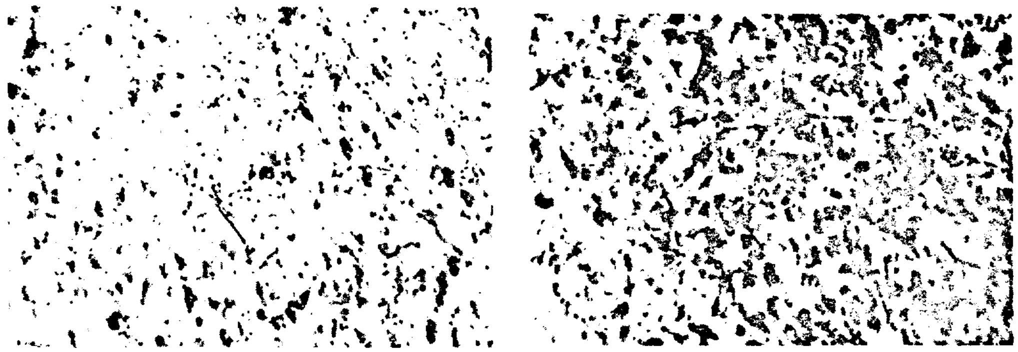

FIG. 2. Magnification of brain area dorso-lateral to the *ventromedial nuclei* in an MSG treated chicken (a), and a saline treated bird (b). Reduced neuron population per definite area is obvious in the damaged area (a) as compared with the undamaged one (b). (×57).

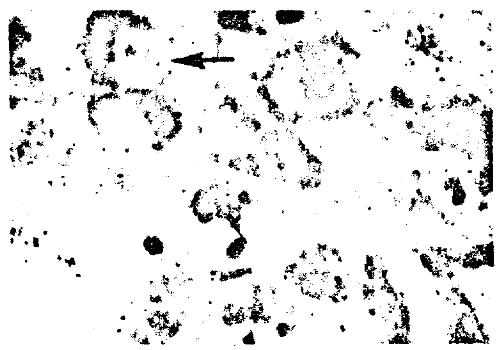

FIG. 3. Necrotic neurons (arrow) in the hypothalamus damaged area (dorso-lateral to the *ventromedial nuclei*). The nuclei of the neuron cells are pyknotic. (×570).

Note: The pictures are representative, and reflect the same results obtained with each MSG dosage used.

the base of the brain including the hypothalamic region (Fig. 1). The damage was so obvious, that it could even be detected with the naked eye. Microscopically, cell density per definite damaged area was found to be much lower than that of the parallel area in the control birds (Fig. 2). Many necrotic neurons could be seen in most of the affected areas (Fig. 3) and their stain absorption, by the cell nuclei, was faint compared with that of the neighboring unaffected areas. The neuron cells' nuclei had distinguished vacuoles, and the whole area was cloudy and swollen. A condensed zone of normal unaffected cells was formed around the damaged area (Fig. 1), possibly as a result of peripheral pressure from the cells of the damaged area.

The described brain damages observed in all the treated groups, were somewhat less obvious in the birds receiving a single injection of 1 mg. MSG/gm. B.W. It should be pointed out that so far the bilateral hypothalamic ventromedial nuclei were unaffected by the MSG treatment. Furthermore, till 45 days of age, neither hyperphagia nor differences in body weight were noted in the treated groups compared with the controls.

The presented observations have shown

that, like some mammals (Olney, 1969; Olney and Sharpe, 1969), the *Gallus domesticus'* central nervous system is also susceptible to damage following treatment with MSG. It should be emphasized, in contrast with the above mentioned studies, that in this one, not all MSG affected neurons were phagocytized and evacuated following treatment. Further studies with birds of varying ages and various MSG dosages are in progress.

SUMMARY

Monosodium glutamate was subcutaneously injected to 5 days old male chicks. The birds were killed at 45 days of age and their brains were histologically observed. The compound induced brain damage in the hypothalamus region surrounding the ventromedial area leaving the latter unaffected. Neither hyperphagia nor increased body-weight over the controls were noted.

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THE RELATIONSHIP OF GLUTAMIC AND ASPARTIC ACIDS TO THE PRODUCTION OF NAUSEA AND VOMITING IN MAN

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In previous work[6] it was found that certain amino acid preparations obtained by the hydrolysis of casein, when administered intravenously to human subjects, produced a depression in voluntary food intake, whereas a mixture of the 10 "essential" amino acids did not cause a reduction in the appetite. Also, during the course of that study, it was observed that if the casein hydrolysates here administered at relatively rapid rates, nausea and vomiting were likely to occur. The observations which form the basis for this report were made in an attempt to determine the contributing factors responsible for these undesirable effects.

It has been reported[2,3,7] that the addition of either glutamic or aspartic acid to known amino acid mixtures, when given intravenously to dogs, greatly reduced the tolerance of these mixtures. Because the mixture of amino acids developed by Madden and Clay[9] and designated by them as "VUJ"* failed to produce anorexia, nausea and vomiting the likelihood of other amino acids being responsible was evident. It was decided to determine whether the dicarboxylic amino acids would produce nausea or vomiting when administered intravenously to human subjects.

A test dose of 0.82 gm. of l-glutamic acid (Merck) dissolved in 500 cc. of an 8% solution of "VUJ" mixture was administered to the first 3 subjects in these investigations. In subsequent studies with other subjects, the amount of glutamic acid was increased to 8.2 gm. dissolved in either 500 cc. of an 8% solution of "VUJ" mixture or in water. In all instances the glutamic acid was partially neutralized with sodium bicarbonate to bring the pH of the mixture approximately to that of the original "VUJ" mixture. The material was steam sterilized before administration.

From calculations based on the nitrogen content of the "VUJ" it was concluded that 1500 cc. of "VUJ" solution (3 bottles) was equivalent to about 100 gm. of protein. Since casein contains approximately 24.6 gm. % glutamic acid,[1] the amino acids derived from 100 gm. of casein would contain about 24.6 gm. of glutamic acid. The dose of 8.2 gm. of glutamic acid per 500 cc. of 8% of "VUJ" mixture is the amount of glutamic acid which would be present in the amino acid mixture "VUJ," if it were derived from casein.

dl-Aspartic acid (Merck) was administered to all the subjects in these experiments at a concentration of 2.1 gm. per 500 cc. of 8% "VUJ" mixture. This amount was likewise based on the estimate that casein contains 6.3% aspartic acid.[1] All samples were steam sterilized before administration. No attempt was made to neutralize the small amount of aspartic acid which was added to the "VUJ" mixture.

The subjects used in this study were ward patients and were chosen on the basis of their willingness to coöperate;

* The amino acid mixture "VUJ" contains the 10 essential amino acids plus glycine and 50% of its amino acids are in racemic form. This mixture was supplied through the courtesy of Merck and Company, Inc., Rahway, N. J.

only those individuals with good veins were selected. During the course of this work some subjects whose voluntary food consumption was being estimated received 3 infusions of the supplemented "VUJ" solution per day for 3 days, while the others received only a single infusion. As a control, unsupplemented "VUJ" solution was administered intravenously to 9 patients. Most of the subjects were given infusions at rates approximating 16 cc. per minute. Due to technical difficulties in administration, a few received the infusions at a slower rate.

TABLE 1.—THE EFFECT OF INTRAVENOUSLY ADMINISTERED GLUTAMIC ACID ON THE PRODUCTION OF NAUSEA OR VOMITING

(The glutamic acid was dissolved in either 500 cc. or "VUJ" or water)

Subject No.	Initials	Sex	Glutamic acid In "VUJ" (gm.)	Glutamic acid In water (gm.)	Rate (cc./min.)	Reaction
1	G. F.*	M	0.82	..	16	Vomited
2	F. C.*	M	0.82	..	17	Nausea
3	F. S.*	M	0.82	..	16	0
			8.20	.	17	0
4	L. B.*	M	8.20	.	As fast as 45	0
5	W. S.*	M	8.20	..	"	0
6	D. O.*	M	8.20	..	"	0
7	M. B.	F	8.20	..	16	Vomited
8	F. D.	M	8.20	..	16	"
9	F. G.	M	8.20	..	16	0
				8.2	16	Dizzy
10	E. E.	M	8.20	..	16	Vomited
11	A. N.	M	8.20	..	16	"
				8.2	16	"
12	A. S.	M	8.20	..	17	0
				8.2	17	Vomited
13	G. S.	M	..	8.2	16	Dizzy
14	K. S.	M	..	8.2	8	0
15	G. C.	M	8.20	..	20	Vomited
16	W. S.	M	8.20	..	12.5	Nausea
17	H. J.	M	8.20	..	12.5	"

* These subjects received 3 doses of the glutamic acid containing "VUJ" for 3 consecutive days.

tolerated 9 doses of "VUJ" fortified with glutamic acid without experiencing either nausea or vomiting. With the administration of this preparation occlusion of the veins was a common occurrence. This was probably due to the hypertonicity of the material since the solution used contained, in addition to the 8% "VUJ" solution, 8.2 gm. of glutamic acid and approximately 2 gm. of sodium bicarbonate. Five subjects received 8.2 gm. of partially neutralized glutamic acid dissolved in water. In this group, 2 subjects vomited and 2 became dizzy. Of the entire group of 17 subjects who received glutamic acid dissolved in either "VUJ" or water, 8 vomited and 3 became nauseated.

Results. Glutamic acid was administered intravenously to 17 patients. The results of this investigation are summarized in Table 1. Of the 3 patients who received 0.82 gm. of glutamic acid dissolved in "VUJ," 1 vomited and another became nauseated. Thirteen subjects were given 8.2 gm. of glutamic acid dissolved in "VUJ" and of these 5 vomited. One subject received both concentrations of glutamic acid and experienced no ill-effects with either. It is of interest that certain patients, such as Nos. 3, 4, 5 and 6 each

The results of the administration of dl-aspartic acid added to 8% "VUJ" solution, are summarized in Table 2. In this series 13 subjects received the test dose; of these, 1 became flushed and 4 complained of nausea.

The 8% "VUJ" solution alone was administered intravenously to 9 patients. These subjects constituted an excellent control group and together they received a total of 41 infusions of 500 cc. each. The rate of administration varied from 17 to 45 cc. per minute. In no instance did vomiting occur and none of the patients in this group complained of nausea.

TABLE 2.—THE EFFECT OF INTRAVENOUSLY ADMINISTERED ASPARTIC ACID ON THE PRODUCTION OF NAUSEA

(The aspartic acid, 2.16 gm., was dissolved in 500 cc. of "VUJ" mixture)

Subject No.	Initials	Sex	Rate (cc./min.)	Reactions
4	L. B.*	M	8	0
5	W. O.*	M	13	0
6	D. O.*	M	15	0
18	I. P.	M	33	0
19	M. S.	M	33	Flushed
20	G. G.	M	20	Nausea
21	O. U.	M	9	0
22	C. C.	M	7	0
23	T. W.	M	17	Nausea
24	B. H.	M	19	0
25	W. S.	M	8	Nausea
26	H. C.	M	25	0
27	A. G.	M	23	Nausea

* These subjects received 3 infusions of "VUJ" containing aspartic acid per day for 3 consecutive days.

Discussion. The amino acid mixture "VUJ", which does not contain any so-called "non-essential" amino acids other than glycine, can be administered at a rapid rate without producing either nausea or vomiting. This mixture presented an ideal vehicle for testing in humans the hypothesis proposed by Madden and his co-workers[2,3] that glutamic and aspartic acids are the factors in protein hydrolysates which reduce the tolerance to these preparations when administered intravenously to dogs. From the observations which form the basis of the present report, it appears that glutamic acid when administered intravenously has a definite tendency to produce nausea and vomiting in man. On the other hand, undesirable reactions occur less frequently when aspartic acid is administered intravenously. It should be pointed out that a smaller amount of aspartic acid than glutamic acid was given. The reason for selecting the doses used in these studies was to obtain the concentrations of these amino acids comparable to those present in the preparations of amino acid mixtures now available for clinical use. The amounts of glutamic and aspartic acids administered were derived by calculation from the data of Block and Bolling[1] on the amino acid content of casein. The quantity of these dicarboxylic acids given was considered to be that which would appear in a casein hydrolysate equivalent to 500 cc. of 8% "VUJ" mixture. It appears that glutamic acid must be administered intravenously to have an emetic effect. Price, Waelsch and Putnam[5] have given 16 to 20 gm. of this acid orally to epileptic patients and only 1 of their 8 cases complained of gastric symptoms.

The data presented here on human subjects are consistent in a qualitative sense with those of Madden and associates[2,3] and also of Unna and Howe,[7] who showed that both glutamic and aspartic acids produced vomiting in dogs.

In 6 patients who received the "VUJ" solution to which glutamic acid was added the influence of this mixture upon the amount of food voluntarily consumed was observed. The method used in determining the influence of amino acid preparations on the voluntary food intake was the same as that previously described.[6] The quantity of glutamic acid administered daily to 4 of these subjects was 24.6 gm. dissolved in 1500 cc. of "VUJ" mixture. The remaining 3 patients received 2.4 gm. in the same volume of "VUJ" mixture. The resulting solution was given intravenously in 3 equally divided doses for 3 consecutive days. One subject received both the high and the low levels of glutamic acid. In 2 of the patients who received the low dose there was a marked decrease in food consumption; 1 vomited violently, the other became nauseated. None of the other subjects developed either anorexia, nausea or vomiting. Thus, it is evident that the depression in food intake may be secondary to the appearance of nausea or vomiting.

Similar observations regarding the influence of aspartic acid upon the voluntary food consumption were made on 3 patients. Each of these patients received 6.3 gm. of aspartic acid dissolved in 1500 cc. of "VUJ" solution. This mixture was given in 3 equally divided infusions for 3 consecutive days. None of these patients showed either a depression of the appetite, nausea or vomiting.

Glutamic and aspartic acids constitute about one-third of the amino acid content of casein. The 2 protein hydrolysates which are most widely employed clinically use casein as the source of protein. These products, therefore, probably have a content of both glutamic and aspartic acids similar to that of casein.* The present work shows that the amino acid mixture "VUJ" which contains no dicarboxylic acids is better tolerated than any of the glutamic acids-containing protein hydrolysates. The hypothesis that glutamic acid and possibly aspartic acid are responsible for the nausea and vomiting in man when protein hydrolysates are given intravenously is supported by the evidence presented in this report. At the present time, however, the possibility of other factors contributing to the undesirable reactions cannot be excluded. Marked flushing, a common complaint due to rapid intravenous administration of protein hydrolysates, was not noted in the patients who received these dicarboxylic amino acid solutions. This undesirable symptom is probably not due to glutamic and aspartic acids.

It is known that psychologic influences are important factors to consider in evaluating the possible emetic effect of a given substance. In all this work the patients were told that the infusion was part of their general routine therapy and they accepted it as such. No leading questions or undue attention was expressed during the procedure. This eliminates to a large degree the possibility of psychic factors causing the nausea or vomiting.

Summary and Conclusions. When glutamic acid dissolved in either the amino acid mixture "VUJ" or water was administered intravenously, nausea or vomiting occurred in 11 out of 17 individuals. Two additional subjects became dizzy.

Aspartic acid was administered under similar conditions to 13 subjects and nausea or vomiting occurred in 4. When the amino acid mixture "VUJ" alone was administered to 9 patients, no undesirable effects occurred.

The probability of the dicarboxylic amino acids being responsible for the decreased tolerance of casein hydrolysates is supported by the evidence presented in this study. However, the possibility of other factors producing nausea and vomiting cannot be excluded.

* "In 'Parenamine' the approximate value of glutamic acid is 24% and of aspartic acid 6% which is the percentage of these non-essential amino acids in casein." This information was supplied by Earl L. Burbidge, M.D., Frederick Stearns & Co., Inc., Detroit, Mich.

No analytic values for glutamic and aspartic acids in "Amigen" are available according to personal communication from Warren M. Cox, Jr., PH.D., at Mead Johnson & Co., Evansville, Ind.

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THE EFFECT OF ORALLY AND INTRAVENOUSLY ADMINISTERED AMINO ACID MIXTURES ON VOLUNTARY FOOD CONSUMPTION IN NORMAL MEN

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Intravenous and oral protein alimentation using hydrolysates of casein for treatment and prevention of protein deficiency states is receiving increasing recognition. Emphasis has been centered around nitrogen metabolism in such surgical conditions as shock (1), burns (2), blood loss (3, 4), wound healing (5, 6, 7), convalescence (8, 9), and postoperative infections (10, 11). Numerous investigations (12 to 15) have shown that positive nitrogen balance can be maintained for short periods of time in patients receiving intravenous amino acid mixtures as the only source of protein. Another of the major indications for parenteral nitrogen feedings is in patients with debilitating diseases who are unable or unwilling to take adequate food by mouth. In such cases the aim is to supplement the oral nitrogen intake by the use of intravenous protein hydrolysates to provide for the restoration of depleted protein reserves and the daily protein requirements.

During the course of previous investigations in this hospital it was observed that individuals who received intravenous injections of an acid hydrolysate of casein ("Parenamine") failed to eat the normal amount of food at the subsequent meal and many complained of loss of appetite. Reports of the occurrence of nausea, vomiting and anorexia associated with the administration of protein hydrolysates are common (12, 16–23). The symptoms of intolerance to these preparations occur most frequently upon rapid injection and constitute one of the disadvantages to their practical use.

Since nausea, vomiting, and anorexia following the infusion of protein digests might result in a decrease in food intake, the advantages offered by intravenous alimentation could thus be offset by a decrease in the consumption of food. The present investigation was undertaken to determine to what extent the administration of solutions of amino acid mixtures would influence the voluntary food intake in normal individuals. Studies were also carried out on the relationship of the rate of injection of amino acid mixtures to the occurrence of anorexia, nausea, and vomiting.

EXPERIMENTAL

Eight normal healthy adult males and 1 patient with portal cirrhosis were chosen for this study. They were permitted to be ambulatory except for the time required to complete the infusions. Each subject was offered a standard diet which supplied 3,544 calories. It contained approximately 135 grams of protein, 180 grams fat and 347 grams carbohydrate. Each item of uneaten food was weighed and the amount consumed was calculated as the difference between the weight of food offered and the weight of that returned. The items in the diet were varied to permit some choice. From the weight of the food consumed it was possible to calculate by the use of standard reference tables (24) the total calories and the amount of fat, carbohydrate, and protein consumed each day.

The general plan of the study was to keep the subject on the diet for a few days, then give amino acid preparations either orally or intravenously along with the diet, and note any effect on the food consumption. The days during which added protein was given will be referred to in this report as the *experimental period;* it lasted from 3 to 7 successive days when the supplemental protein was given orally and from 3 to 6 successive days, usually 3, when it was given intravenously. The subjects were under continuous observation for from 21 to 35 days. Each individual was studied for at least 2 experimental periods each of which was preceded and followed by control periods of not less than 3 days. Three amino acid preparations were used and they will be designated mixture I, II, and III.[1]

[1] Mixture I is an enzymic digest of casein and pork pancreas and is marketed under the trade name "Amigen," Mead Johnson and Co. Mixture II is an acid hydrolysate of casein to which tryptophane is added. It is marketed as "Parenamine" and was supplied through the courtesy of Frederick Stearns and Co. Mixture III is a synthetic amino acid mixture containing the ten essential amino acids to which glycine is added. The amounts used were suggested by Madden, S. C., and Clay, W. A. (J. Exper. Med., 1945, 82, 65) and referred to by them as mixture "VUJ." It was supplied through the courtesy of Merck and Co.

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The sequence of amino acid mixtures used during the successive experimental periods was varied, as shown in Figures 1A, 1B and 2. Two subjects received only one of the mixtures, 7 subjects received 2 mixtures, and 2 of the subjects also received a 10 per cent glucose solution and physiological saline solution interspersed with the solutions of amino acid mixtures in order to obtain information on the effect that these solutions had on the food consumption (Figure 1B). In 4 subjects the effect of using casein hydrolysates by the oral route was investigated for 5 experimental periods and in these studies only mixture I was used.

The amount of mixtures given on each experimental day was equivalent to 100 grams of protein.[2] The volume of the solution administered daily was 1,440 ml. given in three 480-ml. infusions following each meal. Care was taken to arrange infusions so that they would not interfere with meals. The final concentration of amino acid mixture I and II was 8.3 per cent and of mixture III, 8 per cent. The speed of the infusions was purposely varied so that the 480 ml. of fluid was delivered in from 60 to 180 minutes. The rate of protein administration, therefore, varied from 13 grams to 40 grams per hour (Table II).

RESULTS

The results of the influence of oral and intravenous protein supplemental feedings on the amount of food voluntarily consumed during all of the control and experimental periods are summarized on Table I and Figures 1A, 1B, and 2. It can be seen that during the control period when the only source of food was the basal diet, the daily caloric intake remained relatively constant in all subjects. It is also evident that when amino acid Mixture I was given by mouth as the supplemental feeding (Figure 1A) there was little effect on the amount of food eaten. These changes, expressed as calories consumed per day, are interpreted as not being significant. Five subjects received amino acid Mixture I parenterally, and, of these, 4 had little or no depression of the caloric intake. The one patient in whom a depression of appetite occurred (Case No. 2, Figure 1A), had an intake of 67 per cent of the basic control diet. In sharp contrast are the results which occurred in all 7 subjects who received amino acid Mixture II by the intravenous route. There was a constant reduction in the food voluntarily consumed; the average fall was to 61 per cent of the normal consumption with variations ranging from 37.8 per cent to 87 per cent (Table II). This reduction occurred in each of 2 experimental periods in 1 subject (Patient 9, Figure 2) when he was given this preparation. The same results followed the giving of this solution irrespective of whether its use preceded or followed the other 2 mixtures.

TABLE I

The effect of amino acid preparations on voluntary food consumption

Patient no.	Initials	Period of study	Days on study	Average total caloric intake, cal. per day	Food consumption, per cent of control
1	G. S.	Control	16	3,227	
		Preparation I orally	9	3,313	103.6
2	S. T.	Control	34	2,257	
		Preparation I orally	7	2,455	104.5
		Preparation I I.V.	10	1,523	67.6
		Preparation II I.V.	3	853	37.8
		Saline I.V.	3	1,948	86.3
		10 per cent Glucose I.V.	3	2,239	99.5
3	H. W.	Control	17	2,783	
		Preparation I orally	5	2,612	93.6
		Preparation I I.V.	3	2,461	88.3
		Preparation II I.V.	3	1,693	60.8
4	J. B.	Control	22	1,807	
		Preparation I orally	5	1,805	99.9
		Preparation I I.V.	3	1,629	90.6
		Preparation II I.V.	3	793	43.9
5	W. M.	Control	24	2,489	
		Preparation I I.V.	6	2,249	90.7
		Preparation II I.V.	4	1,493	60.2
		Saline I.V.	3	2,285	91.8
		10 per cent Glucose I.V.	3	2,829	111.7
6	E. G.	Control	18	2,073	
		Preparation II I.V.	4	1,600	77.2
		Preparation II I.V.	4	1,241	59.9
7	F. S.	Control	13	3,061	
		Preparation II I.V.	3	1,907	62.4
		Preparation III I.V.	3	3,089	101.0
8	G. E. F.	Control	18	2,402	
		Preparation III I.V.	3	2,706	112.7
		Preparation II I.V.	3	2,081	87.0
9	G. C.	Control	23	3,211	
		Preparation I I.V.	3	3,205	99.8
		Preparation III I.V.	3	2,711	85.0

Amino acid Mixture III was administered to 3 men (Patients 6, 7, and 8, Figure 2) and no measurable change of food consumption occurred during or following the preparation. Ten per cent glucose and physiological saline infusions were

[2] The total nitrogen of each amino acid mixture, based on the data supplied by the manufacturer, was multiplied by 6.25 to give the equivalent of the number of grams of protein.

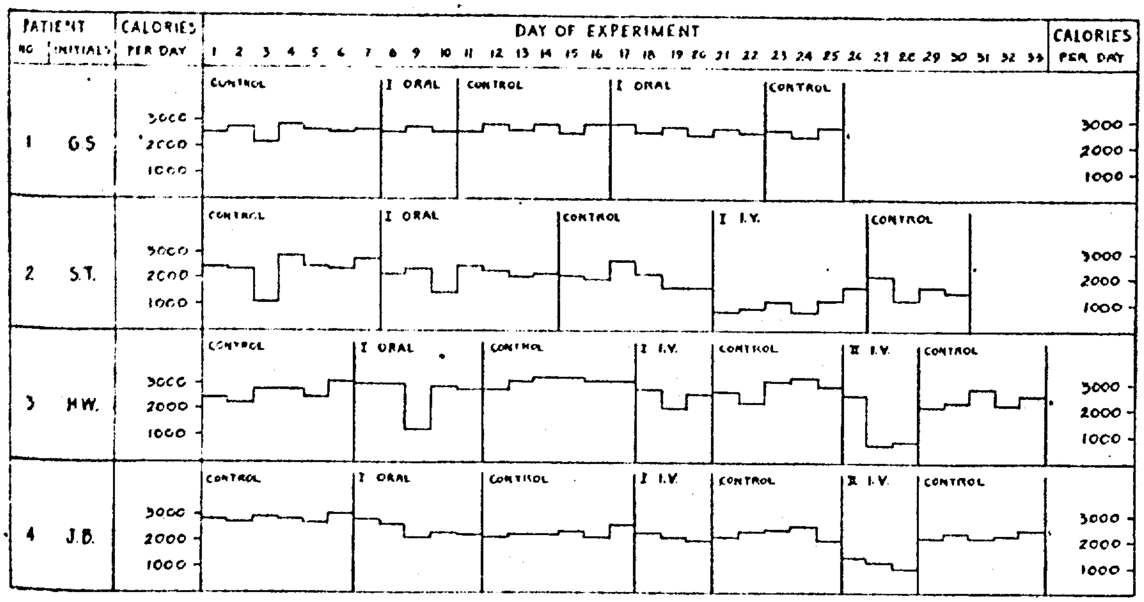

FIG. 1A. EFFECT OF AMINO ACID MIXTURE I ADMINISTERED ORALLY AND INTRAVENOUSLY AND AMINO ACID MIXTURE II ADMINISTERED INTRAVENOUSLY UPON THE VOLUNTARY FOOD INTAKE

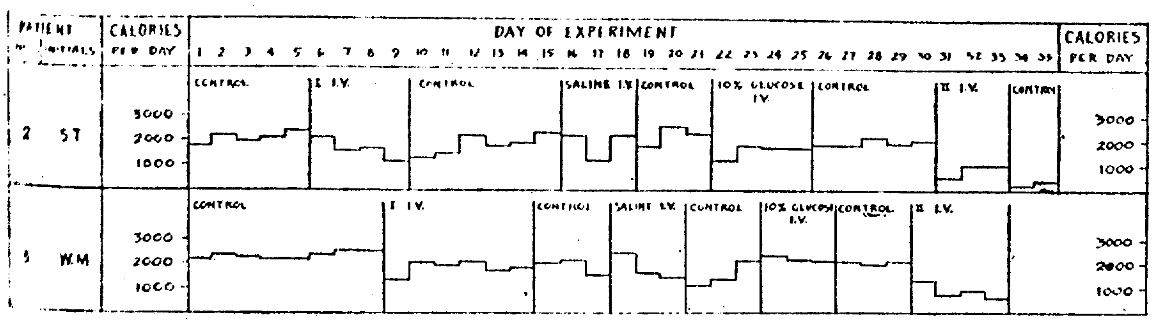

FIG. 1B. EFFECT OF THE INTRAVENOUS ADMINISTRATION OF AMINO ACID MIXTURES I AND II UPON THE VOLUNTARY FOOD INTAKE, COMPARED WITH THE INTRAVENOUS ADMINISTRATION OF PHYSIOLOGICAL SALINE AND TEN PER CENT GLUCOSE SOLUTION

given to 2 individuals and the results are shown in Figure 1B. These 2 commonly employed solutions produced little effect upon the appetite of these men.

In many experimental periods, notably with patients Nos. 2, 4, 5, 6, and 7, there occurred a stepwise depression of food consumption with each additional day during the infusion of supplementary protein. In some instances this progressive depression extended into the first or even the second day of the following control period. This tendency toward a progressive loss of appetite on the last days of the experimental periods and a lag or overlap into the subsequent control period was particularly notable with the use of Mixture II; it occurred to a lesser degree with Mixture I, and was not observed with Mixture III.

It was observed that an important factor which influenced the consumption of food in these subjects was the reactions occurring with or soon after the subject received the amino acid mixture. In the case of amino acid Mixture III no reactions occurred, but Mixture II produced nausea and vomiting in many of the subjects. Another important complaint of the persons receiving the latter preparation was a burning sensation in the arm while receiving the mixture. The burning extended up the arm along the vein to the axilla and was occasionally followed by thrombophlebitis. The thrombosis and pain in the arms made pro-

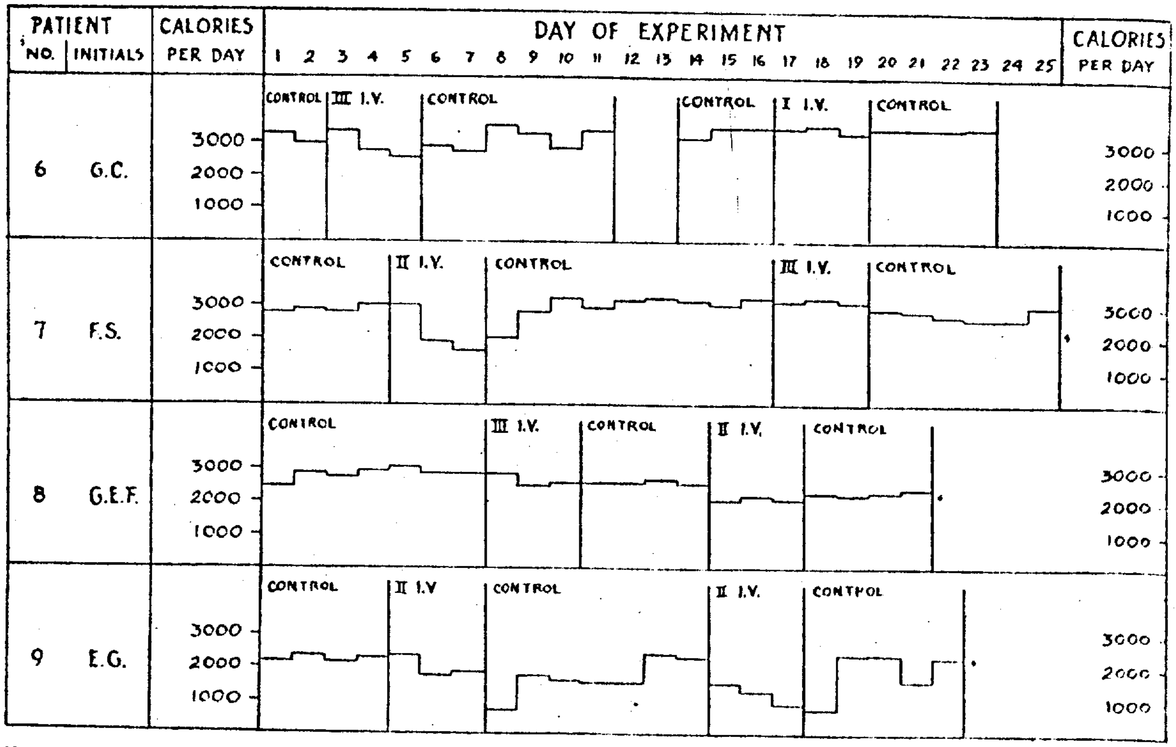

FIG. 2. EFFECT OF INTRAVENOUS ADMINISTRATION OF AMINO ACID MIXTURES I, II, AND III UPON THE VOLUNTARY FOOD INTAKE

longed administration of this mixture difficult. The depression of appetite as reflected in the decreased food intake was not directly related to the presence or absence of the venous thrombosis because the depression occurred in subjects in whom no vein lesions developed, but the associated pain probably contributed to the poor appetite to some extent. Some of the men complained that after receiving any of these preparations they had an unpleasant taste that was similar to the smell of the casein digest. This peculiar taste was reported by a number of patients and remained for a few days after the administration of the amino acids stopped.

TABLE II

Effects of rates of intravenous administration of amino preparation on voluntary food consumption

Patient no.	Initials	Amino acid mixture I				Amino acid mixture II				Amino acid mixture III			
		Rate protein		Change in food intake, per cent of control	Nausea or vomiting	Rate protein		Change in food intake, per cent of control	Nausea or vomiting	Rate protein		Change in food intake, per cent of control	Nausea or vomiting
		grams per hr.	*ml. per min.*			*grams per hr.*	*ml. per min.*			*grams per hr.*	*ml. per min.*		
2	S. T.	13	3.2	−23	N	15	3.7	−63	V				
3	H. W.	40	10.0	−10	*	26	6.5	−37	V				
4	J. B.	22	5.5	−10	*	21	5.3	−57	*				
5	W. M.	24	6.2	−12	*	24	6.2	−39	V				
6	E. G.					30	7.6	−23	*				
						14	3.5	−40	V				
7	F. S.					18	4.5	−38	V	22	5.5	0	0
8	G. E. F.					27	6.7	−13	V	40	10.0	+12	0
9	G. C.	32	8.0	0	0					26	6.5	−15	0

* Information incomplete.

The results of the studies of the relationship of rate of infusion to the food consumption during intravenous protein feedings are summarized in Table II. Some of the peculiarities involved in the rate of administration of these products can be seen in the data derived from patient H. W. (No. 3). Preparation I was given at an average rate equivalent to 40 grams per hour with no ill effects, while Preparation II caused a marked depression in food consumption when given at an average rate of 26 grams of protein per hour. Patient J. B. (No. 4) received both Mixtures I and II at almost identical rates. In this case the drop in calories consumed per day occurred only with the latter mixture. An unusual effect is presented by patient E. G. (No. 9) who received Mixture II on 2 different experimental periods of 3 days each. The rate was 30 grams per hour during the first period and only slight step-wise depression of the total food consumed resulted. At the second experimental period the rate of infusion was approximately ½ that of the first period (14.5 grams per hour) and on this occasion this same mixture caused a pronounced fall in the amount of food eaten. Mixture III did not cause any change in the appetite as judged by the amount of food consumed, and the rate of its infusion was comparable to or faster than those used with the other mixtures.

In these cases where there was some reduction in the voluntary intake after the giving of amino acid digests, the question of selective reduction of any one of the major foodstuffs was investigated. The amount of fat, carbohydrate, and protein eaten by each subject was calculated and expressed as the ratio of Protein: Fat: Carbohydrate. Table III is a summary of some of these ratios. There appears to be no constant decrease in consumption of any given class of foodstuffs after amino acid therapy. Variations in the ratios appear, but no trend is evident. In other words, when these individuals cut down on eating they did not tend to avoid selectively the high protein foods and to eat only foods high in carbohydrates or fats.

DISCUSSION

Co Tui (9) has pointed out that one of the major drawbacks to the clinical use of casein hydrolysates for intravenous protein feedings is the difficulty in giving enough to supply the nitrogen and caloric needs. This is particularly true if it is desired to give large amounts of protein building materials. Elman (25), in presenting a régime for intravenous alimentation of patients suf-

TABLE III

Effect of intravenous amino acid therapy on the ratio of protein: fat: carbohydrate voluntarily consumed
Ratio of protein: fat: carbohydrate of standard diet offered 1:1.32:2.56

Pt. no.	1	2	3	4	5	6	7	8	9
Pt. initials	G. S.	S. T.	H. W.	J. B.	W. M.	E. G.	F. S.	G. E. F.	G. C.
	Fat:CHO*	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO
Control	1:63:2.55	1.38:2.50	1.77:3.00	1.36:3.06	1.34:1.94				
Oral amino acid I	1.39:3.03	1.20:2.45	1.54:2.89	1.42:3.22	1.43:1.66				
Control	1.44:2.60	1.25:2.58	1.54:2.73	1.47:3.22	1.61:1.68				
Oral amino acid I	1.49:2.66								
Control	1.51:2.99								
I.V. amino acid I		1.32:2.66	1.56:3.13	1.57:3.90					
Control		1.37:2.50	1.61:2.98	1.47:3.22					
I.V. amino acid I		1.77:3.70							
Control		1.33:2.49							
I.V. phys. saline		1.28:2.28			1.30:1.70				
Control		1.35:2.42			1.34:1.79				
I.V. 10 per cent glucose		1.52:2.95			1.64:1.83				
Control		1.56:3.20			1.40:1.68	1.47:3.63			
I.V. amino acid II		1.52:1.76	1.92:3.55	1.84:5.35	2.60:2.54	1.51:3.75			
Control				1.55:3.50		1.80:4.00	1.16:2.80	1.39:3.17	1.13:2.43
I.V. amino acid III							1.13:2.40	1.38:3.03	1.15:2.05

* Figures in the table are fat: carbohydrate ratio, protein = 1.

fering from severe protein deficiency, advised the giving of both amino acids and glucose. The sum of the volumes of solutions given per 24-hour period amounted to 3,000 ml., and the total caloric intake was only 1,200 calories, an amount which usually would not be sufficient for a normal subject to maintain his weight. In this investigation the daily dose of the equivalent of 100 grams of protein was arbitrarily chosen because it was considered an amount adequate to provide the generally recommended allowance of 1 gram of protein per kilogram of body weight and a margin to replace depleted protein reserves.

A practical consideration in giving intravenous protein supplements is to employ a method that will not interfere with the patient's daily food intake. In this study 3 injections were given daily, and the individual infusions were completed in not more than 3 hours. Longer infusions were considered inadvisable from the standpoint of comfort to the patient and because the presence of the needle in the vein was a factor which mechanically interfered with the eating of the regular meals. The rates used with Mixture I and II exceeded those usually recommended. Mixture III was frequently given at rates twice those used with the casein digests. The data regarding the rate of infusion in the present study do not permit any final conclusions.

One of the requirements for an acceptable intravenous preparation is that it must be injectable in adequate amounts without producing nausea, vomiting, or other unpleasant reactions. The relationship of reactions which occurred during or following the infusions to the depression of the appetite is a matter of great practical importance. The one constant finding was that, of the 3 mixtures used, the greatest impairment in the appetite occurred during those experimental periods when Mixture II was given. However, it is noteworthy that none of the subjects given Mixture III had any side-effects, and in this same group no significant reduction in the consumption of food was observed. When these same patients received Mixture II impairment of the appetite occurred.

At the present time there is no satisfactory explanation for the occurrence of nausea and vomiting. Albanese (26) questioned if these toxic effects were due to unnatural isomers of certain amino acids which cannot be utilized by the human body. Hopps (27) suggests that the presence of some histamine-like substances, peptones or tyramine as agents may explain the frequently observed reactions of flushing, sensation of warmth, and nausea; but he gives no experimental data to support these ideas. Madden and associates (22) have shown that both glutamic and aspartic acids when added to a mixture of pure amino acids will cause violent vomiting in dogs. The effect of these 2 amino acids upon the voluntary food consumption and as a factor causing emesis in humans is being investigated and will be reported in a separate communication. Hoffman, Kozoll, and Osgood (28) have presented evidence that the height of the amino acid nitrogen content of the blood is probably the factor responsible for producing nausea. They found that if the rate is sufficiently rapid to get a blood amino acid nitrogen value of 10 mgm. per cent or greater, nausea will result. These studies were carried out using Mixture II. Hecht (21), using Mixture II, has suggested that perhaps the rapidity of the rise of the amino acid blood levels and not the actual levels themselves were responsible for the side effects.

The progressive step-wise reduction in the amount of food voluntarily consumed during the succeeding days of the experimental periods occurred with such regularity, particularly with Mixture II, that it must be considered more than a chance occurrence. The overlap into the subsequent first and second days of the control period and the gradual recovery were the usual findings in these cases. At present there is no explanation for this occurrence.

These studies raise the question as to whether the advantages of giving large doses of amino acid mixtures intravenously are offset by increased urinary nitrogen "wastage" which would be expected to accompany the rapid rates of infusion. In the search for a more acceptable preparation for intravenous use, this important point will require investigation.

SUMMARY

The effect of the oral and intravenous administration of mixtures of amino acids on the volun-

tary food consumption in normal human subjects was studied. Three preparations were used: an enzymic hydrolysate of casein (I), an acid hydrolysate of casein (II), and a mixture of the 10 essential amino acids plus glycine (III). The enzymic digest had little effect on the appetite whether it was given orally or intravenously. The acid hydrolysate of casein consistently produced a marked depression in the voluntary food consumption during and following its intravenous administration. The mixture of the essential amino acids had no depressing effect on the appetite. This product was the best tolerated of the 3 tested and could be given at exceedingly rapid rates without any ill effects. When there was reduction in the amount of food eaten, it consisted of a general lack of interest in food rather than a selective rejection of a certain class of foodstuff.

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NUTRITION REPORT INTERNATIONAL

POSTPRANDIAL SERUM AMINO ACID LEVELS IN YOUNG INFANTS FED CASEIN HYDROLYSATE-BASED FORMULAS[1]

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ABSTRACT

A variety of different protein sources are used in infant formulas, including protein hydrolysates. The casein hydrolysates used in certain products contain large amounts of free glutamate and aspartate. Serum free amino acid levels were measured two hours postprandially in one-month-old infants fed a conventional cow's milk-based formula and a casein hydrolysate-based formula to determine the effect of the difference in protein source, and to determine the risk of neurotoxic effects reported to occur in other species after ingestion of high levels of glutamate and aspartate. Although significant differences were noted in serum proline, hydroxproline, alanine, leucine, isoleucine, valine and lysine levels, glutamate and aspartate levels were within normal limits for both groups.

INTRODUCTION

A number of different protein or hydrolyzed protein sources are used as protein bases for infant formulas, and infants fed adequate amounts of these formulas grow normally. Although the changes occurring in plasma amino acid levels in term (1) and premature (2) infants fed varying amounts of the same protein source have been documented, little data are available concerning the effect of variations in the protein source itself on plasma amino acid levels in young infants. Casein hydrolysates are used as the

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protein source of several infant formulas such as Nutramigen[R] and Lofenalac[R] (Mead Johnson) and contain large amounts of glutamate (22% of total) and aspartate (3). The amino acids are present largely in free form in such formulas, and a number of investigators have expressed the concern that the free glutamate may be readily absorbed by the infant ingesting such formulas, with subsequent elevation of plasma glutamate, resulting in neurotoxic effects similar to those occurring in other species after ingestion of high levels of glutamate and aspartate.

A number of investigators reported that suckling mice and rabbits injected with monosodium glutamate or aspartate (250 mg/kg body weight) developed acute and irreversable retinal lesions (4-9). Adult mice were much more resistant to glutamate-induced lesions than newborn animals (7), and glutamate injection of pregnant mice produced no observable abnormalities in the offspring (7). Olney and his collaborators (9-12) have reported that the arcuate nucleus of the hypothalamic region is particularly vulnerable to MSG-induced damage in the infant mouse, rat, rabbit and a single immature rhesus monkey when injected subcutaneously at doses ranging from 0.5 to 2.0 gm/kg body weight. Adamo and Ratner (13) were unable to duplicate these effects in the infant rat, although Arees and Mayer (14) report occurrance of the lesions in adult rats injected with large doses of monosodium glutamate. Olney and Ho (12) recently reported the development of hypothalamic lesions in infant mice after oral ingestion of 3 gm/kg body weight glutamate, aspartate, or cysteine. Thus, lesions result both from oral ingestion and subcutaneous injection.

Although the human infant at birth is a more mature organism than the suckling mouse or rabbit, and the single primate study reported by Olney involved an immature animal injected with an extremely high level of monosodium glutamate (11), these results have rightly led to questions regarding the safety of monosodium glutamate as a food additive, and of infant foods which contain large amounts of glutamate. We have measured plasma and serum amino acid levels two hours postprandially in one-month-old infants fed either a cow's milk protein-based formula or a casein hydrolysate-based formula in an effort to evaluate the risk to human infants from ingestion of glutamate-rich casein hydrolysate-based formulas. Although most studies investigating the effect of diet on plasma amino acid levels utilize fasting samples, we wished to determine the maximum elevation of plasma amino acid levels after ingestion of the glutamate load. Thus a better evaluation is obtained at the time of maximal absorption, approximately two hours postprandially.

SUBJECTS

The subjects were enrolled in a study being carried out in the University of Iowa Pediatric Metabolism Unit, relating the effect of diet to body composition of normal human infants. The infants studied were between 28 to 33 days of age, and had been fed the designated formulas *ad libitum* (providing 67 kcal./100 ml.) at four hour intervals since birth. The selection of infants and the operation of the unit have been described in detail elsewhere (15).

METHODS

Infants of the control group were fed a conventional cow's milk protein-based formula[2], and the test group Nutramigen® (Mead Johnson). Blood samples were drawn approximately 2 hours postprandially, at the same time of day (1 P.M.) to minimize circadian rhythm effects. The chilled specimen was centrifuged, the serum removed and frozen at -50°. The samples were immediately deproteinized when transfered to our laboratory with solid sulfosalicylic acid (16), and the deproteinized sera stored at -70° until assayed. Since cystine is lost in serum samples, plasma samples were obtained from two additional Nutramigen®-fed infants to evaluate the effect of the formula on this amino acid.

Amino acid analyses were carried out on Technicon NC-1 Amino Acid Analyzers using the buffer system described by Efron (16), and the automatic temperature control system developed in this laboratory (17).

RESULTS

Serum free amino acid levels, reported as micromoles per 100 ml., are shown in Table I. No significant elevation of serum glutamate or aspartate levels were noted, although glutamine was significantly lower in the Nutramigen®-fed group. Serum glutamate levels in both groups were slightly greater than the postprandial plasma glutamate levels we have reported earlier for the young infant (18). This is to be expected since some conversion of glutamine to glutamate and pyrrolidone carboxylic acid takes place in frozen sera unless samples are deproteinized immediately (19;20), a condition not possible to accomplish since the samples were originally collected for another study and had been stored a short time at -50° without deproteinization. In addition, cysteine and cystine levels are not reliable in sera since these amino acids are lost both during the clotting process and upon storage in the presence of serum proteins (19,20). Plasma amino acid levels were measured in two additional control infants and Nutramigen®-fed infants to check these levels. Plasma glutamate levels averaged 7 micromoles per 100 ml. and plasma ½ cystine levels averaged 9 micromoles per 100 ml. in both control and Nutramigen®-fed infants. These data indicate that serum glutamate and glutamine levels are not elevated postprandially in Nutramigen®-fed infants, although significant differences in the concentrations of several other amino acids (hydroxyproline, proline, alanine, valine, isoleucine, leucine and lysine) were noted.

DISCUSSION

Despite the relatively large amount of free glutamate (22%) present in the casein hydrolysate-based formula, serum glutamine and glutamate

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[2] The formula used was 3215 A (Mead Johnson). This is the basic Enfamil® formula with 80% corn oil and 20% coconut oil as the fat source.

TABLE I

Postprandial Serum Amino Acid Levels in Young Infants Fed Cow's Milk Protein or Casein Hydrolysate-Based Formulas

Amino Acid	Protein Base: cow's milk n = 24	Protein Base: casein hydrolysate n = 8	t	p
	micromoles per 100 ml*			
Taurine	11.4 ± 4.57	10.2 ± 3.07	0.67	0.5
Hydroxyproline	9.99 ± 2.81	5.36 ± 2.06	4.1	<0.002
Aspartate	1.39 ± 1.19	2.08 ± 1.49	1.3	0.2
Threonine	20.1 ± 3.99	23.4 ± 6.69	1.6	0.1
Serine + Asparagine	24.0 ± 4.71	27.6 ± 5.87	1.6	0.1
Glutamine	63.3 ± 7.61	56.2 ± 6.01	2.3	0.03
Glutamate	9.8 ± 2.80	10.1 ± 2.07	0.27	0.8
Proline	29.5 ± 6.84	39.8 ± 6.41	3.6	0.002
Citrulline	3.51 ± 1.07	3.63 ± 0.89	0.27	0.8
Glycine	34.2 ± 4.87	35.6 ± 8.14	0.59	0.6
Alanine	53.2 ±12.7	69.9 ±10.3	3.2	0.002
α-Aminobutyric	2.05 ± 0.42	2.16 ± 0.38	0.68	0.5
Valine	29.2 ± 5.99	40.2 ± 8.13	3.9	0.002
½ Cystine **	-	-	-	-
Methionine	4.36 ± 1.01	5.70 ± 1.28	2.9	0.01
Isoleucine	9.36 ± 1.70	13.3 ± 1.76	5.0	<0.002
Leucine	19.3 ± 3.38	24.1 ± 2.34	3.6	0.002
Tyrosine	11.5 ± 2.81	9.46 ± 1.96	1.8	0.07
Phenylalanine	10.3 ± 1.48	10.7 ± 1.56	0.56	0.6
Lysine	25.5 ± 5.11	35.4 ± 4.88	4.6	<0.002
Ornithine	18.5 ± 4.74	17.6 ± 3.34	0.50	0.6
Histidine	13.1 ± 1.67	14.0 ± 2.45	1.1	0.3
Arginine	13.6 ± 4.73	16.5 ± 5.43	1.4	0.2

* Expressed as mean ± 1 standard deviation

** Cystine cannot be determined in serum samples levels are not elevated. The decrease in glutamine levels in Nutramigen[R]-fed infants may reflect the high intake of glutamate however, since similar decreases in plasma glutamine have been noted after ingestion of a glutamate load (21). If a substantial overload of glutamate had occurred, a marked increase in serum glutamate would be anticipated. McLaughlan et al. (21) report glutamate levels were increased 3 to 3.5 fold and aspartate levels doubled at peak absorption after ingestion of 0.2 gm/kg body weight monosodium glutamate by the young rat. We have noted similar effects in the newborn pig and monkey after a monosodium glutamate load, and have found that maximal levels occur approximately 2 hours after ingestion if monosodium glutamate is administered in an infant formula (22). Our studies (22) with the newborn pig and monkey indicate that large plasma glutamate elevations (100 to 200 μmoles per 100 ml.) occur when the animals are loaded with the large doses of monosodium glutamate used by Olney and his collaborators (9-12). Thus, the free glutamate in these formulas presents no threat to the young infant.

The significantly increased serum alanine levels in Nutramigen[R]-fed infants may also reflect the glutamate content of the formula. Wiseman and his collaborators (23-25) have reported that substantial quantities of ingested glutamate may be absorbed as alanine. It seems likely that this alanine is formed by a glutamate-dependent transamination of pyruvate, the pyruvate being formed either from carbohydrate via glycolysis, or from glutamate itself after transamination via Kreb's cycle enzymes (α-ketoglutarate → succinyl-coenzyme A, → fumarate, → malate), and conversion of the malate formed to pyruvate via the nicotinamide adenine dinucleotide phosphate malate dehydrogenase.

Although Snyderman et al. (1) have reported that proline and branched chain plasma amino acid concentrations are increased with increased protein intake in the infant, the increased valine, isoleucine and leucine levels in Nutramigen[R]-fed infants are likely not caused by the slightly increased protein content of the casein hydrolysate-based formula (2.2 vs 1.5 gm/100 ml). Plasma amino acid levels in infants fed soy protein-based formulas containing 2 gm/100 ml protein closely resemble those of our control group (26). Snyderman et al. (1) have also studied the effect of a completely synthetic diet, the protein moiety of which was a mixture of 18 L-amino acids, on plasma amino acid levels. At similar protein levels, the plasma amino acid levels of infants fed the synthetic diet did not differ substantially from those in infants fed a conventional cow's milk protein-based formula. However, it should be pointed out that these authors measured plasma amino acid levels 4-5 hours postprandially, thus the influence of diet was largely lost. Due to technical reasons, these authors were unable to calculate glutamine and glutamate values in their samples, and the affect of glutamate levels in the amino acid mixture on plasma glutamine and glutamate levels is not known. Thus, the decreased level of hydroxyproline, and increased levels of valine, isoleucine, leucine, alanine, lysine and proline reflect the composition of the casein hydrolysate formula, and indicate that the protein source has a substantial influence on plasma amino acid levels postprandially.

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Spread of Iontophoretically Injected Ions in a Tissue

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Equations were developed for the spread of ions iontophoretically injected into a tissue, on the assumption that the material is transported by electrical forces. Corrections were made for the uptake by cellular elements and blood vessels of the injected material which tends to move through the extracellular spaces. The predictions made from these equations agreed with the dimensions of tissue changes produced in the rat's cerebral cortex by the iontophoretic injection of glutamate.

1. Introduction

The electrophoretic injection of ions into tissues through micropipettes, first employed by Nastuk (1953), has been a useful tool in physiological investigations. The material thus released has been assumed to remain well localized, and the amount to be proportional to the applied current (Zieglgänsberger, Herz & Teschemacher, 1969). Therefore it has been considered possible to evaluate the effects of the electrophoretic injection of various substances on single cells. This method has been used on spinal neurons by Curtis & Eccles (1958) and by Curtis, Phillis & Watkins (1959, 1960), on brain stem nerve cells by Curtis & Koizumi (1961) and on neurons in the cerebral cortex by Krnjevic & Phillis (1963). Attempts to evaluate the actual amount of ions released have been made by Krnjevic, Mitchell & Szerb (1963), and by Obata, Takeda & Shinozaki (1970), the former using an assay of acetylcholine and the latter a radioisotope technique. Curtis, Perrin & Watkins (1960) calculated the concentration as a function of distance from the microelectrode in a theoretical model based on the assumption that the ions move through the medium by diffusion only. Herz, Zieglgänsberger & Färber (1969) applied a similar model to their study

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of the distribution of iontophoretically injected glutamate and γ-aminobutyric acid in brain tissue. The concept that electrophoretically injected ions move through the tissue by electrical forces will be considered in the present paper as an alternate possibility. The observation of Van Harreveld & Fifkova (1971) that the injection of glutamate in the cerebral cortex causes well defined morphological changes in the tissue offered an opportunity to test experimentally a mathematical model based on the latter assumption.

2. Evaluation of the Area Covered by Electrophoretically Released Ions

In the experimental arrangement under consideration a potential difference is established between a micropipette filled with the compound and an indifferent electrode sufficiently far away so that the electric field established may be assumed to be spherically symmetrical. The tissue is assumed to be a uniform and isotropic medium (Ranck, 1963) in which the applied voltage causes a spherically symmetrical current field, which carries the ions under consideration from the micropipette into the surrounding tissue. The total value of the current is the sum of its component parts

$$I = \sum_{i=1}^{n} t_i I, \tag{1}$$

where $i = 1, \ldots n$ denotes the different ions and t_i is the transference number, or the fraction of the current carried by the ith ion.

The microelectrode and the tissue may be considered as two resistors (R_e and R_t) in series so that the voltage drop across each is

$$V_e = IR_e, \tag{2a}$$

$$V_t = IR_t. \tag{2b}$$

It should be noted that the potential field which causes the ionic movements in the tissue, equation (2b), is dependent only on the current and the resistance of the tissue itself. This field cannot be ignored even though the potential drop in the micropipette, equation (2a), due to its significantly higher resistance may be much larger than that in the tissue (Curtis, Perrin & Watkins, 1960). The resistance of the tissue (R_t) is evaluated by noting that the resistance of a spherical shell with inner radius r and outer radius $r+\mathrm{d}r$ and specific resistance s, is

$$\mathrm{d}R_t = \frac{s\,\mathrm{d}r}{4\pi r^2}. \tag{3a}$$

Therefore the resistance of a region from the rim of the electrode (r_e) to a point, a distance r from the center, is

$$R_t = \int_{r_e}^{r} \frac{s\,\mathrm{d}r}{4\pi r^2} \tag{3b}$$

$$= \frac{s}{4\pi}\left(\frac{1}{r_e} - \frac{1}{r}\right). \tag{3c}$$

The electric field (E) in the tissue, which causes the ions to move is the gradient of the potential drop and may be expressed in terms of the current (I) and the specific resistance of the tissue by applying Ohm's law and combining with equation (3a) thus

$$E = I\frac{\partial R_t}{\partial r} = \frac{Is}{4\pi r^2}. \tag{4}$$

The electrophoretic movement of ions injected into the tissue from a micropipette can be estimated as follows. The amount of ions of species i, expressed as a function of their charge, put into the tissue is equal to the product of the (constant) current, the transference number t_i and the time of application (T); it is also equal to the integral of the charge density of the ions (ρ_i) over the spherical volume covered, thus

$$It_iT = \int_{r_e}^{r} \rho_i 4\pi r^2\,\mathrm{d}r, \tag{5}$$

where r is the radius of the region over which the ions are distributed.

In order to calculate the radius of this region, the charge density of the ions as a function of their position must be evaluated. The density of the current carried by the ion species i (J_i) or the amount of charge carried by this ion through a unit area perpendicular to the current per unit time, is equal to the charge density (ρ_i) times the average velocity (v_i) of the ions,

$$J_i = \frac{t_iI}{4\pi r^2} = \rho_i v_i. \tag{6}$$

The velocity of the particle is determined by the equation of motion, that is the product of the mass and acceleration is equal to the sum of the forces acting on the particle. These are the force due to the electric field (E) and a retarding force which is assumed to be proportional to the velocity,

$$qE - kv = ma, \tag{7a}$$

where q is the charge of the particle, m is its mass and k is the retarding force constant, or

$$m\frac{\mathrm{d}^2r}{\mathrm{d}t^2} + k\frac{\mathrm{d}r}{\mathrm{d}t} = qE. \tag{7b}$$

In a constant electric field a charged particle moves through a uniform linear medium with a constant velocity which can be expressed as

$$v = \frac{qE}{k} = \mu E, \tag{8}$$

where μ is the electric mobility of the ion. The $1/r^2$ dependence of the electric field, equation (4), in this spherical problem makes equation (7b) a non-linear differential equation. However, the acceleration can be assumed to be negligible as a first approximation. Combining equations (8) and (4) yields the velocity as a function of the current and the distance from the electrode,

$$v = \frac{s\mu I}{4\pi r^2}. \tag{9}$$

Note furthermore that if the velocity is proportional to $1/r^2$ the acceleration will be proportional to $1/r^5$ and therefore negligible compared with v at some distance from the micropipette.

The density of ion i which can be obtained by combining equations (6) and (9),

$$\rho_i = \frac{t_i}{\mu s}, \tag{10}$$

is independent of both the position and the current and thus uniform in the entire region invaded by this ion. Finally, using this value, equation (10), for the density, equation (5) can be integrated to yield

$$IT = \frac{4\pi}{3s\mu} r^3. \tag{11}$$

This implies that the radius of the region is proportional to the cube root of the current used in the iontophoresis.

(A) THE EFFECTS OF GLUTAMATE ON CEREBRAL CORTICAL TISSUE

The electrophoretic application of glutamate caused characteristic changes in the cerebral cortex visible with the light microscope (Van Harreveld & Fifkova, 1971). Plate I shows a micrograph of a section cut parallel with the cortical surface through a lesion produced by passing a current of 0·25 μA for 1 hr through a micropipette filled with a 150 mM glutamate solution at pH 7. At this pH the glutamate ions are mostly univalent. The preparation was stained with methylene blue and azure II (Richardson, Jarett & Finke, 1960).

The spherical lesion consisted of a central lightly colored spot which on examination with the electron microscope was found to contain grossly swollen dendrites, presynaptic terminals and glial elements. In this spot

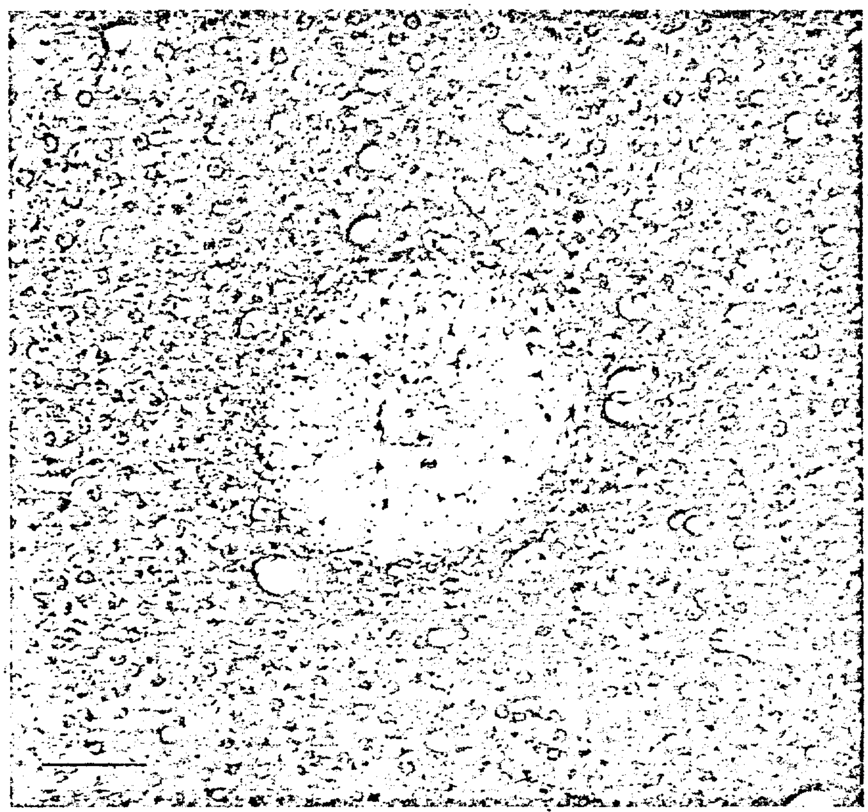

PLATE I. Light micrograph of a lesion caused by the iontophoretic deposition of glutamate in the rat's cerebral cortex. A current of 0·25 μA was applied for 1 hr through a micropipette filled with a 150 mM glutamate solution. The cortex was fixed by perfusion with glutaraldehyde immediately after the glutamate administration, cut parallel with the surface and stained with methylene blue and azure II. The calibration line indicates 100 μm.

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darkly stained, shrunken nerve cells were present. Surrounding the spot was a ring of tissue which was usually somewhat denser than the normal cortical tissue, and contained shrunken nerve cells similar to those found in the center of the lesion. These cells were often surrounded by a lightly stained halo, which in electron micrographs was shown to consist of grossly swollen tissue elements. In this ring furthermore were present small roundish transparent structures which are cross sections of moderately swollen dendrites. Beyond the ring the normal cortical tissue began.

In a number of experiments the current was varied, leaving the duration (1 hr) and concentration of glutamate in the micropipette (150 mM) unchanged. This yielded lesions of varying dimensions. Figure 1 shows that the diameter of the central light spot, which is rather sharply delineated from the ring of dense tissue, is proportional to the cube root of the applied current. This is in agreement with the prediction made above based on the concept that the electrophoretically applied ions are transported through the tissue by electrical forces (equation (11)). A number of other observations, however, were not in agreement with the predictions based on this concept.

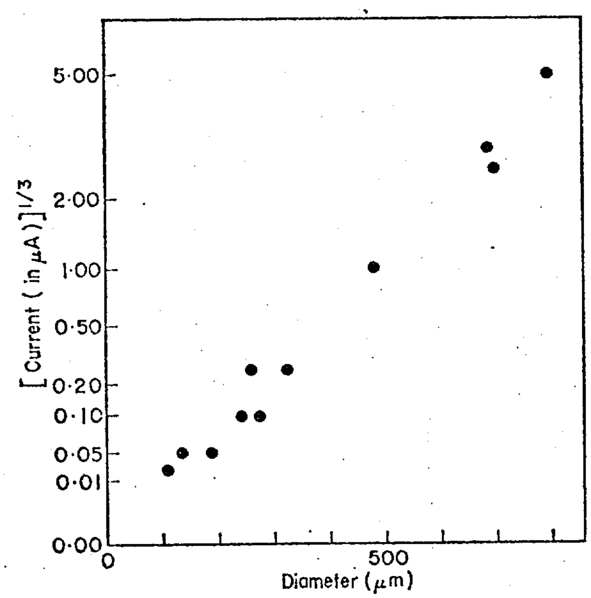

FIG. 1. The cube root of the current (in μA) used for the iontophoretic deposition of glutamate in the rat's cerebral cortex was plotted on the ordinate, the diameter (in μm) of the area in the glutamate lesion characterized by grossly swollen tissue elements on the abscissa. A micropipette filled with 150 mM glutamate was used and the current was applied for 1 hr.

It was concluded above that the concentration of the ions transported from the microelectrode into the tissue is uniform in the entire spot. However, the glutamate lesion showed at least two different appearances, a central region of grossly swollen structures, surrounded by a ring containing less swollen apical dendrites. This suggests that the tissue can respond in two different ways which have different thresholds. The glutamate concentration in the central part can be expected to be high, causing the more pronounced tissue changes. In the ring surrounding the light spot the concentration is subthreshold for this effect but above threshold for milder tissue changes. It is even possible that glutamate spreads further in the tissue but at a concentration subthreshold for any visible reaction.

From the above considerations (equation (11)) it followed furthermore that the diameter of the spot should be proportional to the cube root of the product of current and its time of application (*IT*). The experimental results deviated considerably from this prediction. For instance, by applying a current of 5 μA for 5 min through a micropipette containing 150 mM glutamate a spot was produced with a diameter of 596 μm. The amount of glutamate deposited by this current in 5 min ($1{\cdot}5 \times 10^{-3}$ coulomb) could have been transported by a current of 0·42 μA for 1 hr. From Fig. 1 it can be estimated that such a current would have produced a glutamate spot with a diameter of only about 370 μm.

Since the cellular elements are surrounded by membranes of relatively high resistance most of a current applied to a tissue is carried by extracellular ions (Cole, 1940; Van Harreveld & Ochs, 1956; Van Harreveld, 1966). The glutamate released from the micropipette thus can be expected to move at first mainly in the extracellular spaces. However, from there it may be taken up by the tissue elements and be transported through the vessel walls into the blood. This would result in a decrease in extracellular glutamate concentration toward the periphery of the lesion, explaining the differences in appearance between the center of the glutamate lesion and the ring of tissue surrounding it. Since this removal of glutamate from the extracellular space will take time, a greater loss of the amino acid will occur when it is delivered over an hour than within 5 min. Therefore if the glutamate concentration falls below threshold for the gross swelling at the periphery of the spot, the 1 hr light spot can be expected to be smaller than the 5 min spot.

In some experiments the glutamate concentration in the micropipette was changed by diluting the 150 mM glutamate solution with Ringer's solution. The current was in all instances 0·25 μA applied for 1 hr. The diameter of the glutamate lesion decreased from about 300 to 80 μm when the concentration of the amino acid in the micropipette was reduced from 150 to 10 mM.

The absence of a symbol for the glutamate density in the microelectrode in equation (11) suggests that the spread of the amino acid in the tissue is not affected by this parameter, which is to be expected since the migration of an anion in the electric field is not a function of its concentration. The effect of the glutamate concentration on the diameter of the lesion can be explained by the uptake of the amino acid by tissue elements and blood vessels. This will decrease the glutamate concentration to the threshold concentration for gross swelling at a shorter distance from the electrode tip when the concentration in the micropipette (and extracellular space) is lowered.

3. Correction for Absorption in the Tissue

It was suggested that the discrepancy between the experimental results and the predictions based on the concept of a transport of electrophoretically injected ions into the tissue by electric forces is due to the absorption of glutamate from the extracellular compartment by cellular elements and blood vessels. Assuming that the probability of an ion being removed is proportional to the time it spends in the tissue, then the density at point r is

$$\rho(r) = \rho_0 \, e^{-cT(r)}, \tag{12}$$

where ρ_0 is the density at the orifice of the electrode which is assumed to be equal to that of the solution in the electrode, c is a constant and $T(r)$ the time it takes for a particle to move from the electrode to the position r. This time may be evaluated by integrating equation (9) for the velocity ($v = \mathrm{d}r/\mathrm{d}T$) to give

$$T(r) = \frac{4\pi}{3s\mu I}\,(r^3 - r_e^3). \tag{13}$$

Since the distances travelled are far greater (of the order of 10^2 μm) than the radius of the electrode, the latter term may be neglected, so that the time spent in getting to the point r is proportional to the cube of the distance travelled

$$T(r) = \frac{4\pi}{3s\mu I}\,r^3. \tag{14}$$

The integration of equation (9) is based on the assumption that the specific resistance of the tissue throughout the glutamate spot is uniform. This may not be the case, however, since many factors can affect the tissue resistance such as the uptake of glutamate by cellular elements and blood vessels which may change the specific resistance of the extracellular material, changes in the width of extracellular spaces and of the permeability of cell membranes. These factors cannot be evaluated. However, it will be shown below that the assumption of a uniform specific resistance of the tissue in the glutamate spot leads to an agreement between the theoretical and experimental results. For this reason the integration of equation (9) seems justified.

If T is the length of time the current (I) was applied, then r in equation (14) will be the maximal distance (r_m) traveled by the ions and equation (14) becomes equal to equation (11). In case the concentration does not fall below the threshold for the tissue reaction studied, equation (11) gives the relationship between the size of the spot and the current. If, however, the glutamate concentration falls below the threshold level (ρ_t) the swelling of the tissue elements can no longer occur at the periphery of the glutamate spot, and even though in time T glutamate ions will move a distance r_m, the spot will only be visible up to the point (r_t) of the threshold concentration, so that equation (12) can be rewritten as

$$\rho_t = \rho_o \, e^{-cT(r_t)}, \tag{15}$$

or using equation (14) as

$$\rho_t = \rho_o \, e^{-(4\pi c/3s\mu I)r_t^3}. \tag{16}$$

Taking the ln of equation (16) and rearranging, the equation becomes

$$\ln \rho_o - \ln \rho_t = \frac{4\pi c}{3s\mu I} r_t^3. \tag{17}$$

When the concentration of the compound in the electrode is kept the same, ρ_0 is a constant and so is ρ_t, the threshold for the action on the tissue. Under these conditions r_t and I are the only variables and it follows that the diameter of the spot is still proportional to the cube root of the current, as demonstrated in the series of experiments collected in Fig. 1.

Equation (17) suggests also a linear relationship between the logarithm of the glutamate concentration in the electrode (ρ_0) and the cube of the radius of the glutamate spot. In Fig. 2 the cubes of the diameters of the spot of grossly swollen tissue elements is plotted on the abscissa against $\ln \rho_0$ on the ordinate in a number of experiments in which the glutamate concentration in the microelectrode was varied but the current (0·25 μA) and the time (1 hr) were kept constant. Although the diameter of the spot decreases with the glutamate concentration, the relationship is not linear.

Changes in the concentration of glutamate in the electrode changes the specific resistance of the fluid in the micropipette. The specific resistance of a salt solution can be expressed in terms of density and motility of the ions present ($s = 1/\sum \rho_i \mu_i$). In the present experiment glutamate ions were replaced by faster moving chloride ions while the sodium concentration remained constant and equal to the sum of the anions. The specific resistance

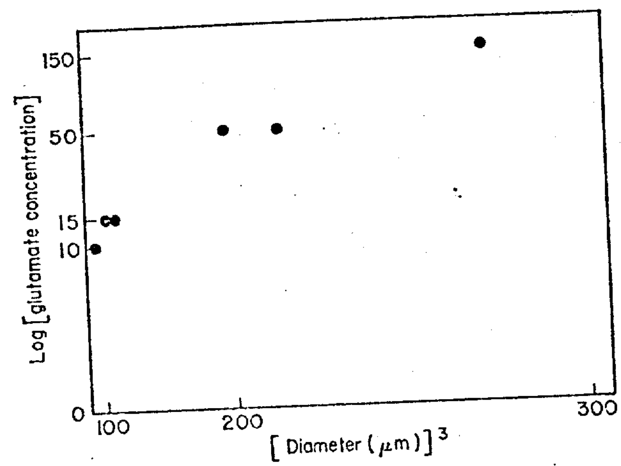

FIG. 2. The logarithm of the glutamate concentration in the micropipette used for the iontophoretic deposition of the amino acid in the rat's cerebral cortex was plotted on the ordinate, the cube of the diameter (in μm) of the area exhibiting grossly swollen tissue elements on the abscissa. The current applied was 0·25 μA, applied for 1 hr.

of the solution can be expressed as

$$s = \frac{1}{\alpha - \beta\rho_G}, \tag{18}$$

where

$$\alpha = \rho_{Na}(\mu_{Na} + \mu_{Cl}),$$
$$\beta = (\mu_{Cl} - \mu_G).$$

The replacement of glutamate by chloride ions thus results in a decrease in specific resistance of the fluid filling the microelectrode. Since the current in the glutamate spot is carried in part by the anions injected from the microelectrode into the extracellular space, a lowering of the glutamate concentration can be expected also to decrease the tissue resistance. Equation (17) can be arranged as

$$s(\ln \rho_o - \ln \rho_t) = \frac{4\pi c}{3\mu I} r_t^3, \tag{19}$$

showing that, because of the effect of the glutamate concentration on the tissue resistance, a change in the density of this amino acid in the microelectrode affects r_t^3 in a non-linear way, causing a larger increment in this value for equal increments of ρ_0 at high than at low concentration of glutamate in the microelectrode in agreement with the experimental findings (Fig. 2). It can be concluded from equation (19) that at the intercept with the ordinate the glutamate concentration in the pipette is at threshold for gross swelling of tissue elements. From Fig. 3 this concentration appears to be of the order of 8 mM.

4. Sufficiency of Electrical Forces

Finally, it will be shown that the electric fields in the physical system are of the proper magnitude to account for the motion of the glutamate ions. The average velocity $\langle v \rangle$ of an ion moving from the electrode to the position r is the distance travelled divided by the time (equation (13))

$$\langle v \rangle = \frac{r - r_e}{[4\pi(r^3 - r_e^3)]/3\mu s I}. \tag{20}$$

The velocity of the ions is proportional to the electric field, equation (8). By combining equations (8) and (20) the average electric field $\langle E \rangle$ between the edge of the electrode and the point r can be expressed as

$$\langle E \rangle = \frac{3sI(r - r_e)}{4\pi(r^3 - r_e^3)}. \tag{21}$$

The specific impedance of normal cortical tissue measured with low frequency (1000 Hz) alternating current is 220–250 Ω cm (Freygang & Landau, 1955; Van Harreveld, Murphy & Nobel, 1963; Ranck, 1963). During asphyxiation and spreading depression the tissue impedance tends to increase markedly. This value is more than doubled a few minutes after the start of the arrest of the blood circulation due to a transport of extracellular material which conducts most of the current used in the impedance measurement into the intracellular space (Van Harreveld & Ochs, 1956). It was postulated that this transport is due to an increase in Na permeability of the plasma membrane caused by a release of intracellular glutamate into the extracellular space (Van Harreveld, 1966). A similar transport of extracellular material can be expected to occur during the iontophoretic glutamate injection, resulting in the swelling of tissue elements. The specific impedance of the tissue in the glutamate lesion will therefore be considerably higher than that of normal cortex. A value of 500 Ω cm seems a fair estimate.

The application of a 0·25 μA current for 1 hr produced a spherical area of grossly swollen tissue elements with a radius of about 150 μm. This was surrounded by a ring of tissue about 100 μm wide showing glutamate effects in the form of swollen apical dendrites and shrunken nerve cells. The total visible spot thus had a radius of about 250 μm. Using equation (21) the average field over this region is 0·05 V/cm using the estimated value for the specific resistance of the tissue in the glutamate lesion. Glutamate was found to move with a velocity of 1·6 μm/sec in a unit field at pH 7·0. The amino acid can thus be expected to move over a distance of 290 μm in 1 hr in an average field of 0·05 V/cm in good agreement with the dimensions of the observed glutamate spot.

5. Discussion

The agreement between the predictions, based on the model in which glutamate ions are transported only by electrical forces, and the experimental findings, suggests that glutamate is indeed distributed in the tissue by electrophoresis. When current is run for only a short period of time, the injected ions can be expected to be distributed with near uniform density throughout a volume proportional to the cube root of the product of the applied current and time of application. For longer periods of current application there may, however, be an appreciable loss of the amino acid from the extracellular material, due to absorption by cellular elements and passage through vessel walls. Since the electric forces are not affected by this disappearance of glutamate ions the region covered is the same as if there were no absorption. However, in this case the density may become subthreshold for a tissue reaction at a smaller distance from the orifice of the microelectrode than the maximum distance over which the ions are transported by the current.

Curtis *et al.* (1960) considered diffusion as the main mechanism for the spread of an iontophoretically injected compound in the tissue. This concept was tested experimentally by Herz *et al.* (1969) who used changes in the discharge rate of nerve cells as an indication of the action of iontophoretically applied glutamate or γ-aminobutyric acid. The amino acids were injected either from an electrode close by or situated at some distance from the recording electrode. Currents of various strength were applied until a response was observed. This occurred after a shorter time when the amino acids were injected from the close electrode than from the remote electrode. The time difference was considered to be a function of the diffusion of the active compounds. Using a modification of an equation of Carslaw & Jaeger (1960) for heat conduction from a point source and suitable values for the parameters of this equation a good fit of the experimental and theoretical data was found.

The experimental conditions of the observations of Herz *et al.* (1969) are quite different from those used in the experiments described above. The current in their experiments was applied for a few seconds through electrodes filled with 3 M glutamate causing a reaction (increase of discharge) with a threshold of 0·25 mM. The concentration difference between glutamate in the electrode and the place where its action is measured is thus of the order of 10^4, highly favoring a spread by diffusion. In the present experiments the current was applied for much longer times (1 hr) and the difference between electrode concentration and the threshold of the tissue reaction (see above) was about twenty-fold. Although the conditions of the present experiments do not exclude a spread of the amino acid by diffusion in addition to that caused by electrical forces, the linear relationship of the dimensions of the glutamate effects and the cube root of the applied current indicate that under the conditions of these experiments electrical forces are dominant in transporting glutamate through the tissue.

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THE MONSTER-PRODUCING ACTION OF GLUTAMIC ACID

By

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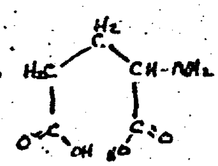
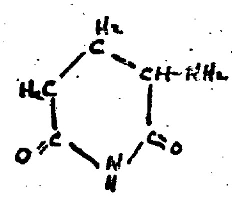
Paper received on 2/29/64

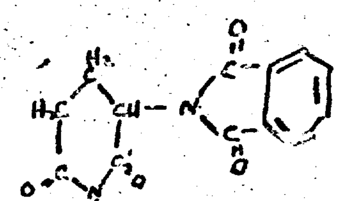
Thalidomide, whose chemical structure is shown below, is a derivative of glutamic acid and exercises a depressive effect on the central nervous system.



Glutamic Acid



Glutarimide



α- Phthalimido glutarimide= Thalidomide

This product, synthesized and studied from the pharmacological viewpoint in 1956, is said to be a sedative and hypnotic, not causing ~~the same~~ fatal toxic effect of strong doses administered by mouth, but certainly carried over to a state of sleep and severe migraine (18, 12, 16, 3). It elevates the threshhold of pain when it is administered with analgesics (10).

It potentializes the effects of depressive substances on the central nervous system, particularly those of the barbiturates, and it antagonizes stimulants of the central nervous system (17, 18,1,13).

Several congenital anomalies have been observed which generally are related to the long bones of new babies born to women who have taken Thalidomide at the beginning of their pregnancy. Also observed have been abortions and fetal resorptions in the case of such women (4,16).

Preparations of glutamic acid (HOOC-(NH_2)CH-CH_2-COOH) are used in our clinic for well-defined therapeutic purposes such as states of hypoacidity, certain types of epilepsy (petit mal), various mental difficulties, etc. (15,6,8,19,1) without mention being made of the relative harm in administering this product to pregnant women or to those who are apt to become pregnant.

Once knowing the effects of Thalidomide on the fetus, we proposed to conduct research on glutamic acid--so commonly employed in different instances--to determine whether or not it is a monster-forming product.

Material and Methods

Experiments have been carried out on 28 rabbits, and at the same time, 28 other rabbits (20 females and 8 males) have served as controls during the experiments. The two groups have been subjected to normal nutritional conditions. The rabbits reserved for experimentation have been divided into three groups.

First Group. 14 rabbits (10 females, 4 males). The females only received glutamic acid. We have taken into account in the administration of this compound the case of females being in a state of pregnancy, and this is the reason only 2 out of 10 females were at the beginning of pregnancy; the other were in a period of pre-pregnancy.

Second Group. 6 rabbits (4 females and 2 males). Both females and males received glutamic acid in combination with vitamin B6 for the following reasons:

a) Glutamic acid is administered in practice to males as well as females, and it is possible to obtain different results in connection with fertilization.

b) Vitamin B6 facilitates the transformation of glutamic acid to GABA and, in the case of a deficiency of this vitamin, it is possible to produce deformities in the following generation in connection with the response of the ovaries to gonadotropic hormones (7,11,22,14).

Third Group. 8 rabbits (6 females and 2 males). Glutamic acid has been administered alone in the case of the two sexes in order to compare the independent effects of vitamin B6. For the third group we have used Glutamidine, which is a specialty pharmaceutical based on the hydrochloride of glutamic acid, administered by mouth in doses of 25 mg/kg in the form of a pellet in caoutchouc.

We prefer the "by mouth" method because of the poor solubility of Thalidomide and glutamic acid in water and because ~~its~~ this therapeutic course is the one most frequently employed. Absorption of the product during its passage through the gastro-intestinal tract is rather easily accomplished.

Glutamidine has been used during a period of 40 ~~hours~~ days administering the product during 4 and 1 ~~hour~~ day intervals, being a a total of 27 hours, at a rate of 25 mg/kg., vitamin B6 at the rate of 25 mg/kg subcutaneously.

At the conclusion of the experiment, the treated animals and their offspring have been observed from a morphological point of view, from an anatomic-pathological viewpoint, and by x-ray of all of the organs, systems, bones, and endocrine glands.

Results

First Group. The autopsy of 10 female and 4 male animals which had continued to live a normal communal life showed, at the end of the period of administration of glutamic acid, that 2 rabbits were in a state of pregnancy but the pregnancy was "arrested". At the uterine level there were diverse calcareous deposits in the region corresponding to the endometrium and infiltration of these elements as far as the myometrium. Finally, there was found a degenerated fetus in the interior of the uterus.

In the case of 2 other rabbits, there was found post-abortive endometritis of the uterus.

The abortus observed with Glutamidine (Fig. 1) corresponds to the observations with Thalidomide (4).

Two rabbits were delivered of young at a normal time, but the offspring had various malformations (Fig. 2). In the case of the two rabbits who did not deliver, hyperplastic uteri were found.

Regarding the young from this group, no pregnancies were observed during 7 months in the case of these offspring subjected to communal life two months after their birth. But we have been able to observe at the end of this period: a number of monstrosities particularly of the extremities, which were long or short, of pathological form and position, and which caused the animals to use these organs with great difficulty (Fig. 3). This observation has been the same in the case of newly-born animals in the other groups subjected to the experiment. Curvature of the spine has been observed and accentuated in proportion to growth (Fig. 4)(4). The growth and development are sharply retarded compared to the contral animals (Fig. 5).

Anatomic-pathological examination has shown serious atrophy of the adrenal capsules, hyperplasia of the adrenal capsules, increase in the basophilic cells of the adenohypophysis, atrophy of the testes, and a cessation of spermatogenesis, and atrophy of the uterine endometrium.

Second Group. Composed of 4 female rabbits and 2 males living a communal life.

Two rabbits gave birth and their offspring showed monstrosities in the form of deformation of the extremities. (Fig.5). The three offspring died during their first hours; no abortions were observed in the animals of this group.

Anatomic-pathological examination of the animals having received glutamic acid showed: hyperplasia of the uterus and inhibition of spermatogenesis.

In the case of the offspring: Hyperplasia of the adrenal glands, increase in basophilic cells of the adenohypophysis, serious atrophy of the adipose tissue, atrophy of the testicles, arrestation of spermatogenesis, and atrophy of the uterine endometrium.

Third Group. Composed of six females and two males who lived a normal communal life.

Three rabbits were delivered of young. Distortions of the extremities and growth impairment was observed (Figure 8(. The other three rabbits did not deliver young. Pathological examination of the animals receiving the medication showed: uterine hyperplasia and inhibition of spermatogenesis in the case of testicles.

In the case of the offspring:

Serious atrophy of the adipose tissue, hyperplasia of the adrenals, atrophy of the endometrium of the uterus, atrophy of the testicles, and arrestation of spermatogenesis. Radiological examination of the long bones showed them to be more slender, their development mediocre, local deformities of the tendons, differences in symmetry of the two extremities of the same animal (Fig. 9(.

Autopsy of the young revealed almost total disappearance of adipose tissue of the whole organism.

Discussion

Our object was to investigate whether glutamic acid, of which ~~is a derivative of~~ Thalidomide is a derivative, has a monster-producing effect analogous to that of Thalidomide (4,20). Glatamidine was administered by mouth in doses of 25 mg/kg to three different groups of rabbits.

In the first group, Glutamidine was administered only to the females. It was this group which presented the anomalies resembling most those of Thalidomide when Glutamidine was administered in this manner to females in a pregnant condition.

Abortion and the arrest of pregnancy was observed only in this group.

We have thought that this state of affairs perhaps was a result of the presence of enzymatic transformation products of glutamic acid in the blood of father and mother (17), being a result of myotic intoxication (4).

Regarding the following generation, one observes mediocre growth of the long bones, a lengthening or shortening in comparison to the normal, hyperplasia of the adrenals and of the uterus, an atrophy of the testes, etc. These observations can arise by an alteration of the activity of oxaloacetic-glutamic transaminase and of pyruvic-glutamic transaminase on the organs of the systems (2,5) and the same on the genes, being an augmentation of the basophylic cells of the adenohypophysis (2,5,21,3).

This reasoning is confirmed by the fact that in the case of hereditary muscular atrophy, the long bones are shorter in comparison with the normal and the activity of oxaloacetic-glutamic transaminase is augmented. Never-the less, one might--with equal justification--think of these modifications of enzymatic activity (still not determined at this time) and also explain the observed results.

In the case of the offspring of the animals of the first group, congenital anomalies and malformations have been observed which (since they have been encountered in the two other groups) have been interpreted as being the toxic and monster-forming effect of glutamic acid administered over a long period.

In the second group, Glutamidine has been administered in association with vitamin B6 to males as well as females. Abortions and arrest of pregnancy at its onset has not been observed, but malformations and pathological results have been the same as those observed in the first and third groups.

In the third group, Glutamidine alone was administered to males and females, but the results parallel those of the second group in the case of the mothers and their young. Also the experiments have not revealed any beneficial or preventitive effect with the compound alone compared to administered in association with vitamin B6.

The effect of Glutamidine on the bone marrow merited a particular examination.

The harmful effects have not been observed on the nervous tissue of the mothers and offspring except as hyperemia. Hyperemia of all of the organs of the animals of the three groups arises as the inhibitive effect of glutamic acid on thrombokinase (8,9).

Disappearance of almost all fatty tissue of the offspring in all groups is due to the adsorption of glutamic acid to the lipid fraction of the liver and to the serum lipoproteins, being a mobilization of the lipids following the metabolism of glutamic acid itself, and an enzymatic transformation of the level of the fatty tissue (9) and finally a direct action on lipogenesis.

Inhibition of growth is related to augmentation of the basophils of the adenohypophysis (10).

The extent of mortality of the offspring and its relation to the organ malformations is distinctly greater than that of the controls.

The main difference between the groups is evident when Glutamidine is administered to males at the same time it is given to females (first group).

In comparing reproduction between the control rabbits and the three groups comprising these experiments, one can observe that the amount of growth in the case of the latter is 30% less than that of the controls.

k This result perhaps can be explained by inhibition of spermatogenesis and by endometrial hyperplasia.

Malformations of the offspring of the second and third groups are less accentuated than those of the first group.

These experiments are presented as evidence of the monster-forming effect of glutamic acid and foreshadow modifications in future generations of those receiving the product over a period of time which causes the effects of this substance on spermatogenesis, on the uterus, and on the adrenals and the hypophysis.

Resume

We have investigated the monster-forming effects of glutamic acid administered over a continuing period to parents and to their offspring, and we have used the rabbit has the experimental animal.

Summary

The administration of glutamic acid (25 mg/kg, per os) for one month to breeding rabbits was found to provoke the following changes: (1) in the parents: hyperplasia of the adrenals and endometrium, inhibition of spermatogenesis, generalized hyperemia, lowered reproduction rate, resorption of the foetus and abortion; (2) in the youngs: hyperplasia of the adrenals, increased basophils concentration in the adenohypophysis, atrophy of the endometrium and of the testes, blockade of spermatogenesis, atrophy of fat tissues, muscular anomalies, atrophy of bone tissue, resulting in malformations of limb bones and poor growth.

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CAPTIONS FOR FIGURES

1. Dead fetus observed from an animal which had taken glutamic acid in continuing doses of 25 mg/kg/day (orally).

2. Malformations observed in the case of offspring of female rabbit on continuing doses of glutamic acid of 25 mg/kg/day, orally.

3. Hind extremity 1.5 times longer and unusable of offspring of mother receiving continuing doses of glutamic acid of 25 mg/kg/day.

4. Curvature of the spine during growth of a small deformed rabbit whose mother received continuing doses of glutamic acid of 25 mg/kg/day.

5. Left. Malformed hind extremities of a small rabbit born of parents receiving glutamic acid and vitamin B6 in continuing doses of 25 mg/kg/day. Right. Small rabbit born of the same group and showing malformations of the front paws.

6. Differences in development of baby rabbit (at left) born of parents receiving glutamic acid and vitamin B6 (25 mg/kg/day) compared with normal animal (right).

7. Radiological examination of the long bone of the control animal (right) and of offspring from parents receiving continuing doses of glutamic acid of 25 mg/kg/day.

8. Mediocre growth of offspring arising from parents receiving continuing doses of glutamic acid of 25 mg/kg/day.

9. Radiological comparison of the control animal (right) with rabbit of parents receiving glutamic acid at rate of 25 mg/kg/day.

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CHINESE-RESTAURANT SYNDROME: RECURRENCE

To the Editor: Recent reports claiming that high doses of monosodium L-glutamate (MSG) may have toxic effects on the central nervous system of animals[1] and evidence that the burning sensations, facial pressure, headache and chest pains of the Chinese-restaurant syndrome are due to monosodium L-glutamate[2] have led to criticism of the widespread use of MSG as a food additive. We recently encountered a case of the Chinese-restaurant syndrome in a 62-year-old epileptic woman who was being treated with diphenylhydantoin (Dilantin), 100 mg three times a day. Because the low serum folate that may occur in patients on diphenylhydantoin therapy[3] has been attributed to an inhibition of intestinal conjugase,[4] which probably splits folate polyglutamate into the easily absorbed mono-L-glutamate form,[5] we suggest that patients on diphenylhydantoin therapy may be more susceptible to the toxic effects of monosodium L-glutamate in the diet. The absorption of folic acid probably involves an active transport mechanism[6] unless vast supraphysiologic quantities are given,[7] and since the dietary folate mainly consists of polyglutamate forms[8] that cannot be absorbed,[9,10] there may well be a deficiency of L-monoglutamates in the gut, the active transport mechanisms for folate L-monoglutamates may be unsaturated, and a dose of monosodium L-glutamate may be rapidly absorbed, the toxic effects of this drug appearing to be dose related.[2] The central effects of diphenylhydantoin and folate deficiency might complicate the response to MSG.

Although more studies are needed, at present it seems reasonable to advise patients on diphenylhydantoin to avoid foods rich in MSG, especially wonton soup.

We should be very grateful for any information about other cases of the Chinese-restaurant syndrome in patients on diphenylhydantoin therapy.

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Light- and Electron-microscopic Changes in Central Nervous Tissue after Electrophoretic Injection of Glutamate

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The electrophoretic injection of glutamate into the cerebral cortex of the rat caused a lightly stained area consisting of grossly swollen tissue elements in light-microscope preparations. In electron micrographs some of these structures could be identified as presynaptic terminals and dendrites. Also perinuclear and perivascular glial material showed gross swelling. The nerve cells in the lesion were heavily stained in the light- and electron-dense in the electron micrographs. This area was surrounded by a ring of dense tissue containing darkly stained nerve cells and moderately swollen apical dendrites. The magnitude of the spot of grossly swollen tissue elements was found to be proportional to the cube root of the current used for iontophoresis. The size of this spot was also a function of the glutamate concentration in the micropipette used to deposit the amino acid. The swelling of the tissue elements was reversible, but the dark nerve cells did not recover and were removed. The similarity between the "dark" cells in preparations of central nervous tissue ascribed by several authors to mechanical damage of the material, and the dark cells caused by the application of glutamate was pointed out.

The large impedance increase recorded during spreading depression and asphyxiation of central nervous tissue suggested that a major movement of electrolytes and water from the extracellular space into the intracellular compartment occurs under these circumstances (Leão and Ferreira, 1953; Freygang and Landau, 1955; Van Harreveld and Ochs, 1956, 1957; Ranck, 1964; Van Harreveld, 1966). More direct evidence was supplied by the demonstration of a transport of chloride and water into cellular structures during spreading depression and asphyxiation (Van Harreveld, 1957, 1958, 1961; Van Harreveld and Schadé, 1959) and a reduction of the extracellular space observed in electron micrographs (Van Harreveld, Crowell and Malhotra, 1965; Van Harreveld and Malhotra, 1966, 1967; Van Harreveld and Khattab, 1967; Van Harreveld and Steiner, 1970). It has been suggested that this movement of extracellular material into the intracellular compartment is caused by an increase in sodium permeability of the plasma membrane of susceptible structures (Van Harreveld and Ochs, 1956; Van Harreveld, 1966) and that the increase in membrane permeability is the result of a release of glutamate from the intra- into the extracellular compartment (Van Harreveld, 1959, 1966, 1970). The latter postulate was supported by several observations. Spreading depression could be elicited by the topical application of glutamate in relatively low concentration (15 mM) to the cerebral cortex (Van

Harreveld, 1959). In chickens, before the development of the blood–brain barrier, intravenously administered glutamate caused in the corpus striatum the features of spreading depression and asphyxiation, such as a depression of the electroencephalogram, a slow potential change and an impedance increase of the tissue (Fifkova and Van Harreveld, 1970). Ames (1956, 1958) demonstrated an uptake of sodium, chloride, and water by rabbit retinas bathed in a medium containing glutamate. In electron micrographs of turtle and mouse retinas a large swelling of (dendritic) elements was observed in glutamate-treated preparations (Wald and de Robertis, 1961; Van Harreveld and Khattab, 1968a). Furthermore a release of glutamate from the isolated retina was demonstrated during spreading depression (Van Harreveld and Fifkova, 1970).

In the present investigation glutamate was deposited electrophoretically from a microelectrode in the cerebral cortex and the results were investigated with the light and electron microscopes.

METHODS

Glutamate was injected electrophoretically from glass micropipettes drawn of Pyrex tubing with an outside diameter of about 1 mm. The electrodes were filled by boiling under vacuum with an isotonic (150 mM) sodium 1-glutamate solution (pH 7.4) or with dilutions of this solution with Ringer's solution. Microelectrodes filled with Ringer's solution were used as controls. Depending on the current to be used for electrophoresis (5–0.02 μA) their resistance was adjusted to 5–50 $M\Omega$ by cutting off the tip of the electrode. The desired current, measured with a calibrated galvanometer, was obtained by adjusting the applied potential (microelectrode negative).

In rats anesthetized with urethane an area of the parietal bone, 1.5–2 mm in diameter, was removed with a high-speed dental drill without injuring the dura. The tip of the electrode mounted on a microdrive was placed under guidance of a binocular microscope on the dura. After piercing the dura the tip was lowered about 0.5 mm deep into the cortex.

The cortex was fixed by perfusion from the abdominal aorta (Van Harreveld and Khattab, 1968b) with 2.5% glutaraldehyde in an 0.15 M phosphate buffer (pH 7.4). The frontal part of the rat was in this way perfused with 250 ml of the fixative under a pressure of 13 cm of mercury. For light microscopy a block (about 4 × 4 mm) was cut from the cortex at the location of the micropipette. After dehydration it was embedded in paraffin and cut parallel with the cortical surface. The sections were stained with methylene blue and azure II (Richardson *et al.*, 1960). For electron microscopy similar but smaller blocks were postfixed at 4°C with 1% OsO_4 in the phosphate buffer, dehydrated for 2 hours in repeatedly changed acetone and passed through propylene oxide for 1 hour. The blocks were left overnight in a 50% mixture of Epon and propylene oxide and then embedded in the plastic. Polymerizing was achieved by keeping the preparation for 2 days at room temperature, then 2 days at 37°, 1 day at 45°, and 2 days at 60°. Thick sections (10 μ) were cut parallel to the surface on a rotary microtome and stained as the paraffin sections to locate the glutamate lesion. The block was then trimmed and thin sections were cut on an LKB Ultrotome, stained with lead citrate (Reynolds, 1963), and viewed with a Philips 200 electron microscope.

RESULTS

The glutamate effect

Typical glutamate effects were produced by passing a current of 0.25 μA for 1 hour through a micropipette filled with an isotonic (150 mM) glutamate solution introduced about 0.5 mm deep into the rat's cerebral cortex, well within the cell layers. A charge of 9×10^{-4} C passed through the pipette in 60 minutes. Since the charge on an equivalent of ions is 9.65×10^{4} C and less than half the charges are carried by glutamate ions the amount of glutamate deposited was of the order of 5×10^{-9} mole, or less than 1 μg.

Light microscopy of cortex fixed directly after the end of such a glutamate application and sectioned parallel with the cortical surface showed a region of grossly swollen structures (Fig. 3), which made this area stand out as a light spot about 300 μ in diameter. In between the swollen structures were cells which stained more darkly than those in the normal tissue and appeared shrunken. This spot was surrounded by a ring of tissue which had a somewhat denser appearance than the normal tissue. The ring also contained shrunken, darkly stained cells which were surrounded by a transparent halo, and a large number of small transparent structures often present in groups, which may be cross sections of moderately swollen apical dendrites. The transition between the light spot of grossly swollen structures and the denser ring was rather sharp.

Electron micrographs of the central area of the glutamate lesion showed, as expected, numerous grossly enlarged, electron-transparent structures (Figs. 12–19). Some of these could be identified as presynaptic endings by the vesicles they enclosed which were often concentrated at the region of the synaptic membrane thickening (Figs. 17–19). Many of these endings contained swollen mitochondria. Other distended structures formed synaptic contacts with presynaptic endings, characterizing them as dendritic in nature (Fig. 18). However, many of the swollen elements could not be recognized because of the lack of an identifying structure. It is likely that some were glial components since the perivascular and perinuclear glial cytoplasm, which can readily be identified, was also grossly enlarged (Figs. 14, 15). Often ruptures of plasma membranes were observed (Fig. 13) which may have occurred either during the glutamate application or during the fixation of the distended structures. The thin nonmyelinated axons and the myelinated fibers never showed signs of swelling and appeared normal (Figs. 12, 19).

The cells in the glutamate spot were severely affected. Electron-dense cells containing vacuoles, mitochondria, and an array of endoplasmic reticulum and ribosomes, which seemed to have been derived from the Nissl substance, were present (Figs. 16, 17). Synaptic contacts with axonal endings identified them as somata of nerve cells (Fig. 17). Such cells were surrounded by enormously swollen structures which usually could not be identified (Fig. 16). The latter constitute the halos around shrunken cells in light micrographs which were especially prominent in the ring surrounding the spot of grossly swollen elements (Fig. 3). Small, dark structures, often found in between swollen elements, could sometimes be identified as dendrites by synaptic contacts (Fig. 19). It seems likely that these dark dendrites are processes of the electron-dense nerve cells.

Glia cells did not exhibit the electron dense, shrunken appearance of the nerve cells. On the contrary, the perinuclear cytoplasm (Fig. 14) and perivascular endfeet (Fig. 15) exhibited gross swelling and also the smaller glial elements in the neuropil may have been enlarged. The glial nucleus, however, maintained a rather normal appearance (Fig. 14).

Control experiments

Two kinds of control experiments were carried out. In one, a microelectrode filled with Ringer's solution was introduced into the cortex and currents of 0.25–5 μA were passed through it for 1 hour. Some of the resulting preparations showed no tissue changes of any kind. In others the electrode had produced some injury which showed up as a small bleeding or as a few swollen tissue elements. In another set of experiments a microelectrode filled with 150 mM glutamate was introduced into the cortex but no current was passed. In some instances there were no tissue changes, in others the signs of tissue injury mentioned above were found. A small area of swollen tissue elements was observed only in one case in which the tip of the electrode had been cut off. It seems likely that in this instance the opening of the micropipette was so large that sufficient glutamate diffused out to cause the tissue reaction. To obtain the typical glutamate lesion described above it was thus necessary to pass current through a glutamate-filled microelectrode.

Effect of the strength of the electrophoretic current

In a series of experiments currents of 5–0.02 μA were passed for 1 hour through micropipettes filled with 150 mM glutamate. The cortex was fixed immediately at the conclusion of the glutamate administration. The appearance of the glutamate spot was in all cases the same: an area of grossly swollen structures surrounded by a region of denser tissue containing darkly stained, shrunken cells and numerous small transparent tissue elements (apical dendrites). The diameters of the glutamate spots varied with the strength of the current. Figure 1 shows that a linear relationship existed between the cube root of the current and the diameter of the spot of grossly swollen elements. The amount of glutamate delivered by the electrode is proportional to the product of time and current. If the glutamate deposited in 1 hour were distributed evenly over a spherical area around the electrode tip, the linear relationship between the cube root of the current and the diameter of the spot would follow.

Figure 9 shows a glutamate lesion produced by applying a current of 5 μA for 5 minutes through a micropipette containing 150 mM glutamate. This spot, which featured grossly swollen tissue elements, had a diameter of 596 μ. The amount of glutamate deposited by this current (1.5×10^{-3} C) could have been delivered by a current of 0.42 μA for 1 hour. From Fig. 1 it can be concluded that such a current would have produced a spot of grossly swollen cellular structures of only about 370 μ in diameter. This discrepancy may be explained as follows. Since current in a tissue is almost exclusively carried by extracellular ions (Van Harreveld and Ochs, 1956) the glutamate moves mainly through the extracellular spaces. From there it may be taken up by the cellular elements in the spot and also may

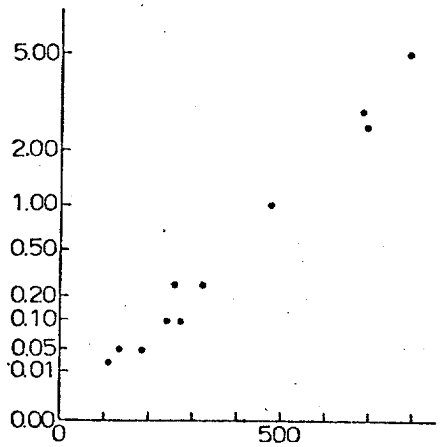

FIG. 1. The cube root of the current (in μA) used for the iontophoretic deposition of glutamate in the rat's cerebral cortex is plotted on the ordinate. A micropipette containing 150 mM of the amino acid was used and the current was applied for 1 hour. On the abscissa is plotted the diameter (in μ) of the area in the glutamate lesion characterized by grossly swollen tissue elements.

diffuse through the vessel wall into the blood. Assuming that this loss of glutamate is a function of the time that the amino acid travels in the nervous tissue, a greater loss will be incurred by the same amount of glutamate delivered over an hour than during a 5-minute period. If the glutamate concentration falls below the threshold for the gross swelling of tissue elements at the periphery of the spot, the 1-hour spot can be expected to be smaller than the 5-minute spot.

Effect of the glutamate concentration in the micropipette

In another series of experiments the micropipettes were filled with an isotonic glutamate solution and with mixtures of this with Ringer's solution resulting in final glutamate concentrations in the micropipettes of from 150 to 10 mM. The current was in all instances 0.25 μA for 1 hour. The cortex was fixed directly after the glutamate administration. The spots formed by pipettes filled with the various solutions were very much alike in general appearance, consisting of an area of swollen structures surrounded by dense tissue with shrunken nerve cells (Fig. 10), but differed in diameter. Figure 2 shows the relationship between the glutamate concentration in the pipettes and the diameters of the areas of gross swelling. Since the glutamate concentration in the tissue cannot be expected to be greater than in the pipette the threshold for gross swelling seems to be below 10 mM.

The amount of glutamate delivered by the microelectrode is determined in addition to current strength and time of application, by the transference number of the amino acid which is a function of the glutamate concentration in the mixture of a solution of this compound and Ringer's solution in the pipette. Less glutamate is thus released by the pipettes filled with 50 mM than those with 150 mM of the amino acid, and even less by the 10- and 15-mM pipettes. The loss of the amino acid to the tissue elements and blood vessels may, therefore, reduce the

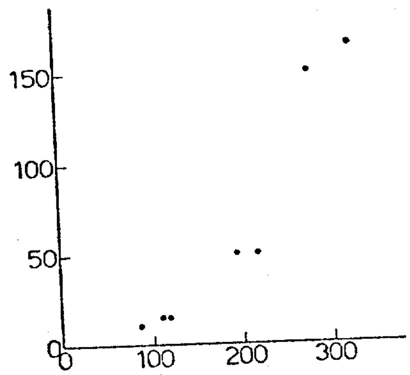

FIG. 2. The glutamate concentration (in mM) in the micropipette used for the deposition of this amino acid is plotted on the ordinate. The current was 0.25 μA, applied for 1 hr. On the abscissa is plotted the diameter (in μ) of the area in the glutamate lesion characterized by grossly swollen tissue elements.

concentration below threshold for the gross swelling most quickly with the 10-mM pipette, accounting for the difference in magnitude of the glutamate spots in these experiments.

Recovery of the glutamate lesion

In a series of experiments glutamate was deposited in the cortex by a current of 0.25 μA for 1 hour from a pipette containing 150 mM of the amino acid. Afterward the wounds were sutured and the animals received penicillin when the recovery was followed for periods longer than 24 hours. After intervals varying from 6 hours to 15 days the rats were again narcotized with urethane and the brain was fixed by glutaraldehyde perfusion for light and electron microscopy.

Recovery for 6 hours. Figure 4 is a light micrograph of a glutamate spot in cortex fixed 6 hours after iontophoresis. The lesion was smaller than that shown in Fig. 3 which was fixed immediately after the glutamate application. Swelling of the individual tissue elements had decreased somewhat. In the spot and in the ring of tissue immediately surrounding it were dark, shrunken cells. Furthermore, numerous small transparent structures, probably cross sections of swollen dendrites, were still present in the ring.

The electron microscope showed numerous grossly swollen elements; some of these were swollen presynaptic terminals, others were dendritic structures. Small electron-dense structures were found, sometimes making contact with presynaptic terminals revealing their dendritic nature (Fig. 20). There was still grossly swollen perinuclear and perivascular glia, although these structures did not appear as devoid of inclusions as after immediate fixation. The profiles of small nonmyelinated fibers were not swollen and appeared quite normal.

Recovery for 24 hours. Figure 5 shows that the glutamate lesion 24 hours after application of the amino acid still appeared in the light microscope as a light spot consisting of swollen tissue elements and shrunken dark cells surrounded by a ring of tissue which contained cross sections of many small transparent structures (swollen apical dendrites).

In the electron micrographs of a glutamate spot fixed after 24 hours there were still enlarged dendrites (Figs. 21, 23) which often showed very distinct microtubules. Also enlarged presynaptic structures were still present although many had returned to the dimensions and electron density usually found in glutaraldehyde-fixed material (Figs. 21, 23). The perivascular glia was less swollen and contained glycogen granules (Fig. 22). Similar granules were found in the glial processes in the neuropil and may be present in dendritic structures (Fig. 21). There were myelinated fibers which showed a wealth of inclusions, mostly mitochondria (Fig. 24), others appeared normal.

Recovery for 2 and 3 days. Figure 6 is a light micrograph of a glutamate lesion in cortex fixed 3 days after iontophoresis. The glutamate spot had lost its lightly colored appearance because it contained no grossly swollen structures any more. However, there were many more cross sections of small transparent structures present in the spot than in the surrounding tissue. Many dark cells were found in the spot.

The electron micrographs of the lesion fixed after 2 days still showed electron-transparent dendrites, and presynaptic terminals with a very uneven distribution of the synaptic vesicles which has been interpreted as an indication of swelling (Van Harreveld and Khattab, 1968b). However, the grossly swollen structures observed during the first 24 hours after the glutamate application had disappeared. Enlarged electron-transparent dendritic structures and electron-dense elements, probably also of dendritic nature, were observed in close contact with or surrounded by glial cytoplasm containing glycogen granules. It was surmised that these structures were in the process of being destroyed by the glia (Figs. 26, 28).

After an interval of 3 days the swollen dendritic structures and presynaptic terminals had almost returned to the dimensions expected in glutaraldehyde-fixed material. There were still present opaque dendrites and electron-dense nerve cells which contained numerous ribosomes, and were surrounded by electron-transparent structures (Fig. 25). Although enlarged, the latter structures were less swollen than those found in ems of cortex fixed immediately after the glutamate application (Fig. 16). The swelling of the perinuclear glial cytoplasm had regressed and it now contained more inclusions (Fig. 25). Also, the swelling of the perivascular glia had disappeared. The nonmyelinated small axons looked quite normal (Fig. 27).

Recovery for 1 week. The glutamate lesion in light micrographs still contained small transparent structures (Fig. 7). The number of cells seemed to have increased as compared with the glutamate spot after 3 days, possibly due to glial cells which migrated into the tissue.

The ems of the glutamate spot after an interval of 1 week showed dendrites which contained unusually distinct microtubules. The latter sometimes exhibited an uneven distribution which may be indicative of moderate swelling (Fig. 29). The presynaptic terminals showed mostly a more or less uniform distribution of synaptic vesicles although in some the vesicles were aggregated on one side of the structure. The slight to moderate swelling suggested by the uneven distribution of microtubules and synaptic vesicles may have been due to the fixation by glutaraldehyde perfusion which itself causes an uptake of extracellular material by dendrites and probably also by presynaptic terminals (Van Harreveld and Khattab, 1968b; Van Harreveld and Steiner, 1970). Remnants of the electron-dense nerve cells and dendrites were in close connection with or enclosed by glial cytoplasm, often containing glycogen granules (Figs. 30, 31, 32). Some glial elements (reactive astrocytes) contained numerous fibrils. The perivascular glia which had regained its usual electron density contained many ribosomes, sometimes arrayed on the endoplasmic reticulum. Cellular structures (macrophages) containing transparent, round vacuoles which may have been fat drops, were often found in the immediate vicinity of the vessels (Fig. 30). The vacuoles were so large that they could be seen with the light microscope. In addition to normal myelinated fibers some grossly abnormal axons were present.

Recovery for 2 weeks. The density of the glutamate spot was similar to that of the surrounding tissue in light micrographs of cortex allowed 2 weeks for recovery (Fig. 8). There were cross sections of many small transparent tissue elements in this area. No normal-appearing nerve cells were found in the spot, and the number of small dark cells had declined as compared with the tissue fixed after 1 week.

The ems of a glutamate lesion fixed 2 weeks after iontophoresis were not greatly different from those perfused after a 1-week interval. The microtubules in the dendrites were still quite distinct. Reactive astrocytes were present, as were remnants of electron-dense nerve cells and dendrites.

Effects of damage to the cortex

In preliminary experiments the dura, intact in the results reported above, was removed before the introduction of the microelectrode into the cortex. This resulted frequently in minor damage to the brain tissue and the pial vessels. Preparations of these cortices examined with the light microscope often showed irregular lightly stained lesions which resembled the glutamate spots. Numerous cellular elements were swollen, although less severely than those in the center of the glutamate lesion. Furthermore, these areas were characterized by dark, shrunken cells surrounded by a light halo (Fig. 11).

Electron micrographs of this region exhibited similar features as found in the glutamate spots. Electron-dense nerve cells, with endoplasmic reticulum and ribosomes reminiscent of the Nissl substance, were surrounded by enlarged tissue elements. Such structures may correspond to the dark cells surrounded by a light halo seen in light-microscope preparations (Fig. 11). There was swelling of pre- and postsynaptic structures as well as of perivascular glia. Broken membranes were often seen. These tissue changes, although very similar, were less severe than those observed in glutamate spots.

DISCUSSION

It was suggested that at the periphery of the area in which grossly swollen tissue elements were found the glutamate concentration is threshold for this particular reaction. The glutamate may spread beyond this boundary, however, where it could be above threshold concentration for other reactions of the tissue.

The center of the glutamate spot which contains the grossly swollen dendritic elements, presynaptic structures and glia, and in addition, shrunken nerve cells is surrounded by a ring of less severely altered tissue which contains shrunken cells and many small transparent tissue elements. In the normal tissue around the glutamate lesion cross sections of apical dendrites appear as roundish, transparent structures often bunched together in groups in sections cut parallel with the cortical surface. The small transparent tissue elements in the ring resemble these apical dendrites although they are somewhat larger and tend to be present in greater numbers. It is suggested that these elements are apical dendrites which are moderately swollen. Dendrites so small that they remain unnoticed in the normal tissue may in this way become visible. These findings could be explained by the assumption that the threshold concentration of glutamate for cell shrinkage and swelling of apical dendrites is lower than that for the gross swelling of the tissue elements in the center of the spot. Such preparations were obtained after a relatively long (1 hour) electrophoretic application of the amino acid (Fig. 3). When a comparable amount of glutamate was deposited in a much shorter time (5 minutes) no distinction of a central spot of grossly swollen dendrites with a ring of tissue containing shrunken cells could be made (Fig. 9). By applying the amino acid in a short time a more equal distribution of glutamate in the affected area can be expected since there is no time for an appreciable loss of the amino acid from the tissue due to absorption by cellular elements and diffusion through the vessel walls. The concentration in such experiments may, therefore, remain above threshold for the gross swelling of tissue elements in the entire area through which the amino acid spreads.

It seems evident that the gross swelling of the tissue elements in the center of the glutamate lesion cannot have been accomplished by uptake of the local extracellular material. It seems possible that the surrounding tissue supplied some of this material. This might account for the ring of denser tissue which often surrounds the central glutamate spot (Fig. 3). It is also possible that part or all of the material which made the gross swelling of tissue elements possible was derived from the blood vessels.

It has been postulated that the anatomical changes observed during asphyxiation (and spreading depression) of central nervous tissue are due to a release of glutamate from the intracellular compartment (Van Harreveld, 1959, 1966, 1970). Although the effects of electrophoretic injection of glutamate into cerebral cortical tissue have a certain resemblance to those of asphyxiation, there are some differences. In the center of the glutamate spot there is a gross swelling of dendritic, presynaptic, and glial elements, whereas asphyxiation and spreading depression cause only a more moderate swelling of apical dendrites (Van Harreveld, 1957, 1958). It is possible that this difference is caused by differences in the glutamate concentrations in the tissue under these circumstances. This possibility is supported by the presence of numerous transparent structures, probably cross sections of moderately swollen apical dendrites, in the ring of tissue surrounding the center of grossly swollen elements where the glutamate concentration can be expected to be smaller. Another difference is the presence of dark shrunken cells in and around the center of the glutamate spot. Asphyxiation was found to cause a transport of water into the somata of nerve cells (Van Harreveld, 1957), whereas the glutamate injection caused cell shrinkage. This may also be due to differences in glutamate concentrations under the two sets of circumstances.

It is of interest that the numerous nonmyelinated nerve fibers present in the cortical tissue are not affected by glutamate application. Also asphyxiation and spreading depression never resulted in swelling of nonmyelinated axons (Van Harreveld *et al.*, 1965; Van Harreveld and Malhotra, 1967; Van Harreveld and Steiner, 1970). Some myelinated fibers in glutamate spots fixed some time after iontophoresis exhibited a normal appearance, others showed gross changes (Fig. 24). The former may have been axons just passing through the spot, whereas the latter may have arisen from cells in the glutamate lesion which are affected by the amino acid.

Although there are differences in the histological and electron-microscopic changes caused by asphyxiation and spreading depression and by glutamate injection, they may be explained by differences in the concentration of the amino acid in the tissue. The effects of glutamate injection seem to support in general the thesis that the tissue changes caused by asphyxiation (Van Harreveld *et al.*, 1965; Van Harreveld and Malhotra, 1966, 1967; Van Harreveld and Steiner, 1970), spreading depression (Van Harreveld and Khattab, 1967) and possibly by fixation with glutaraldehyde (Van Harreveld and Khattab, 1968b; Van Harreveld and Steiner, 1970) are due to a release of glutamate from the intracellular into the extracellular compartment.

It is interesting that nerve cells and some dendritic elements became shrunken and electron dense whereas other dendrites became swollen and electron transparent. It can be surmised that the dark dendrites are processes of the dark shrunken cells located in the glutamate lesion, whereas the swollen dendrites are apical dendrites of cells situated in the deeper layers of the cortex which are not directly affected by the amino acid. This was supported by preparations of cortices, cut at right angles with the surface, in which darkly stained apical dendrites were observed to arise from dark, shrunken nerve cells. The changes in the nerve cell and dark dendrites are clearly deleterious. They often are contacted or enclosed by glial elements, suggesting that they are being phagocytized and removed. In the glutamate spot 2 weeks after the glutamate application no normal nerve cells were present. The present observations are in good agreement with the reports of a serious deleterious effect of systemically administered glutamate on central nervous tissue. Orally or intravenously administered glutamate can reach the tissue only as long as the blood–brain barrier is not developed. When given to infant mice this amino acid caused irreparable degeneration of nerve cells as first observed by Lucas and Newhouse (1957) in the retina. Acute neuronal necrosis in several regions of the infant mouse and monkey brain were described by Olney (1969) and Olney and Sharpe (1969). The necrosis of nerve cells observed under these circumstances may be identical with the effect of electrophoretic glutamate administration observed in the present experiments.

With the exception of the effects on the nerve cells, the results of glutamate administration to the cortical tissue are reversible. The grossly swollen tissue elements in the center of the spot tend to regain more normal dimensions, although this may take several days. This is perhaps not surprising since most of these nervous elements are derived from somas situated outside the glutamate lesion and, therefore, not directly affected by the amino acid.

Several authors have described "dark" neurons in light-microscope preparations of central nervous tissue. Cammermeyer (1962) reviewed the extensive literature and presented evidence that such dark cells are the result of mechanical damage to the tissue. Mugnaini (1965) and Cohen and Pappas (1969) showed that the dark cells appear in ems as electron-dense neurons. These cells resemble the dark neurons in glutamate spots, including the presence of a "shrinkage space" surrounding the cell, which corresponds to the halo in the present paper. The electron microscope showed that this halo is not a space, but consists of grossly swollen tissue elements. A comparison with the light- as well as with the electron microscope confirmed the identity of the dark cells caused by tissue damage in the present paper with those produced by iontophoretic glutamate injection, although the changes tended to be more pronounced in the latter. This observation suggests a mechanism for the formation of dark cells in damaged tissue. Mechanical deformation may release glutamate from the intracellular compartment, as also seems to occur during asphyxiation, spreading depression and fixation. Since the latter are not characterized by the presence of dark cells it can be surmised that mechanical damage tends to cause a greater glutamate release than asphyxiation and fixation, resulting in a concentration of the amino acid in the extracellular spaces high enough to result in the formation of dark cells.

The mechanism by which glutamate causes the changes which result in the dark cells is obscure. However, a suggestion can be offered for the formation of the halo. Glutamate application to the retina causes a release of labeled glutamate with which the tissue has previously been charged (Van Harreveld and Fifkova, 1970). Such a process with positive feedback may result in the build-up of a high concentration of the amino acid around the damaged neurons, which in turn may lead to gross swelling of the tissue elements in the neighborhood of the cell, thus creating the halos.

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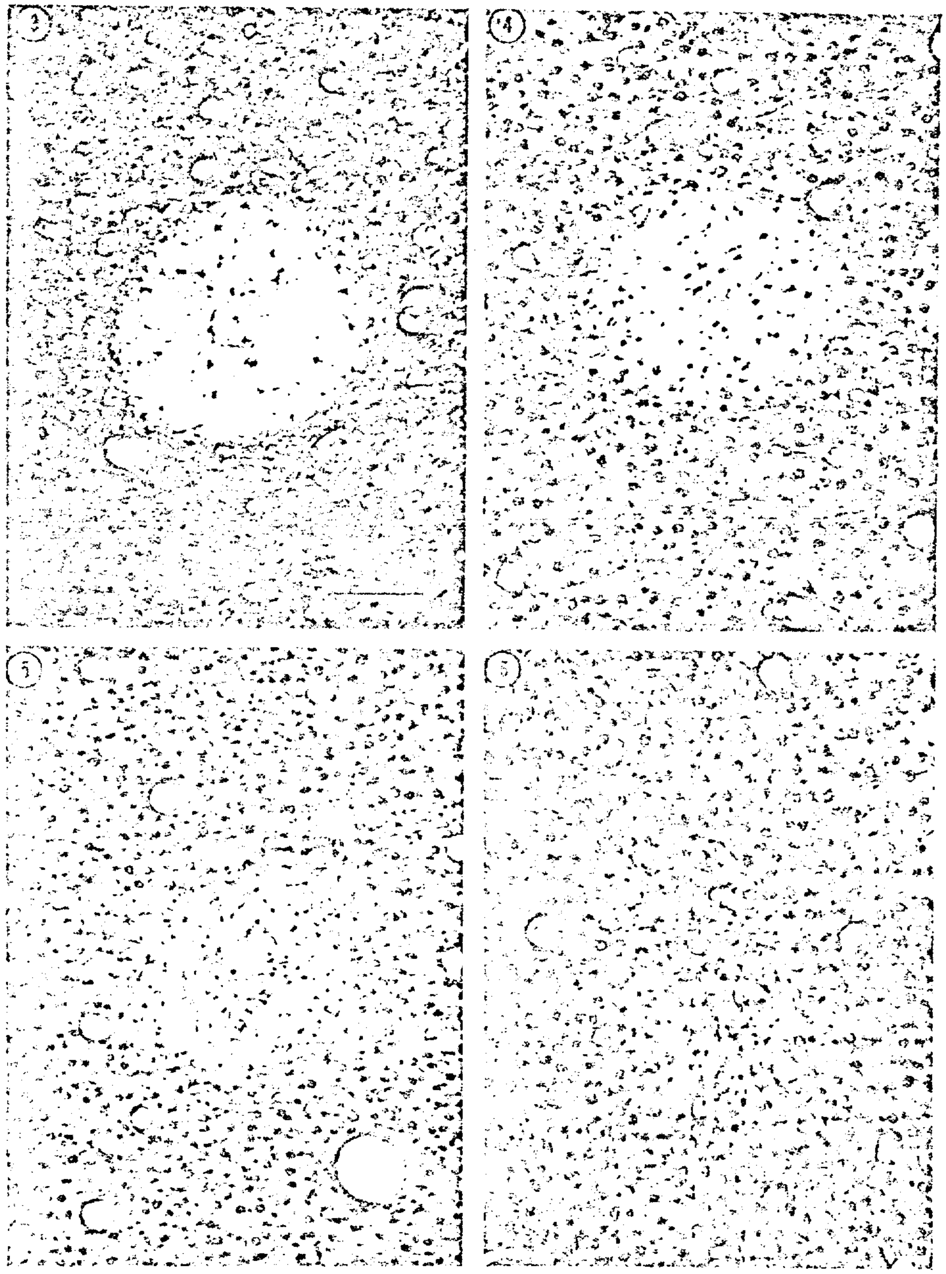


FIGS. 3-6. Light micrographs of rat cerebral cortex. The tissue changes were produced by the electrophoretic injection of glutamate. In all instances a current of 0.25 μA was applied for 1 hour through a micropipette filled with 150 mM glutamate. The cortex shown in Fig. 3 was fixed by perfusion with glutaraldehyde immediately after the glutamate application, the tissue in Fig. 4 was fixed 6 hours after the end of the injection, the cortices shown in Figs. 5 and 6 were perfused after 12 hours and 3 days, respectively. The calibration line in Fig. 3 indicates 100 μ.

Figs. 7 and 8. Light micrographs of the effect of glutamate application as described for Figs. 3-6 (0.25 μA for 1 hour, through a micropipette filled with 150 mM glutamate). The cortex shown in Fig. 7 was fixed 1 week, that in Fig. 8 was perfused 2 weeks after iontophoresis. The calibration line in Fig. 7 indicates 100 μ.

Fig. 9. Light micrograph of cortex into which glutamate was injected from a pipette containing 150 mM of the amino acid. The current was 5 μA, the duration 5 minutes. The magnification is given by the calibration line in Fig. 7 (100 μ).

Fig. 10. Light micrograph of cortex into which glutamate was injected from a micropipette containing 15 mM of the amino acid (made isotonic with the Ringer salts). The current was 0.25 μA, the duration 1 hour. The magnification is the same as in Fig. 7.

Fig. 11. A cortical region is shown with changes due to damage of the cortex and pial vessels. The cortex was perfused with glutaraldehyde 1 hour after inflicting the damage. The magnification is the same as in Fig. 7.

Figures 12–32 show electron micrographs of cortex affected by an electrophoretic glutamate injection. In all the experiments yielding these ems the micropipette was filled with 150 mM glutamate, and a current of 0.25 μA was applied for 1 hour. However, the interval between the end of the injection and fixation by glutaraldehyde perfusion was different. In Figs. 12–15 the cortex was fixed immediately after the injection.

The following abbreviations are used in Figs. 12–32. A, nonmyelinated axons and axon fields; BV, blood vessel; CP, cytoplasm; D, dendrites; ER, endoplasmic reticulum; G, glia; M, mitochondria; MA, myelinated axon; MT, microtubules; N, nucleus; PO, postsynaptic structures; PR, presynaptic terminal; SV, synaptic vesicles; V, vacuole.

Fig. 12. Enormously swollen, electron-transparent tissue elements, some of which can be identified as presynaptic terminals by the synaptic vesicles they contain. In addition to these swollen elements there are much smaller structures, which are less electron-transparent, probably nonmyelinated axons. Also, a myelinated nerve fiber is present. The calibration line indicates 1 μ.

Fig. 13. Break in a plasma membrane (indicated by the arrow); such breaks are frequently observed in this material. The calibration line is 0.5 μ.

Fig. 14. Glia cell with well-preserved nucleus. The two components of the nuclear membrane show a varying separation. The cytoplasm looks rather normal in some regions, in others it is grossly swollen and electron-transparent. Calibration 1 μ.

Fig. 15. Capillary surrounded by perivascular glia which is partly enormously swollen. Calibration 5 μ.

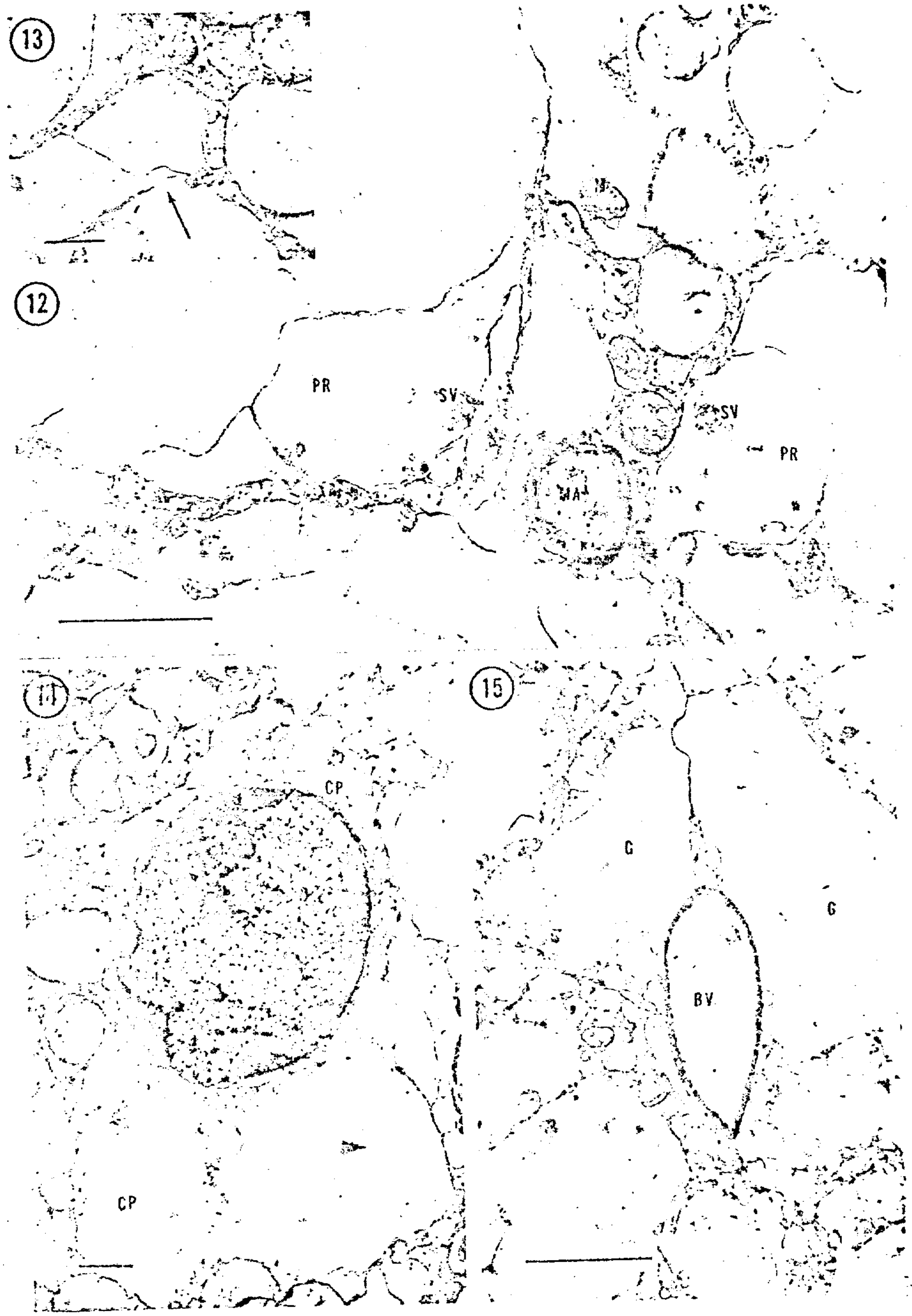
Figs. 16–19. Electron micrographs of glutamate-injected cortex perfused with glutaraldehyde immediately after the application of the amino acid.

Fig. 16. Shrunken electron-opaque nerve cell or large dendrite containing some vacuoles and mitochondria. This structure is surrounded by grossly swollen elements which cannot be identified. They contain distended mitochondria and endoplasmic reticulum. The calibration line is 1 μ.

Fig. 17. Another shrunken nerve cell at higher magnification. It contains vacuoles and ribosomes in a configuration resembling that of Nissl substance. A presynaptic terminal forms a synapse with this cell. The calibration line indicates 0.5 μ.

Fig. 18. An enlarged presynaptic terminal containing synaptic vesicles and a mitochondrion forms a synapse with a grossly swollen dendritic structure which contains swollen mitochondria. The calibration line indicates 0.5 μ.

Fig. 19. Swollen presynaptic terminals, two of which synapse with an electron-dense structure probably a dendritic branch of a shrunken nerve cell. Other grossly swollen structures are present which cannot be identified. Some nonmyelinated axons do not show any swelling. Calibration 0.5 μ.



FIGS. 12 to 15.

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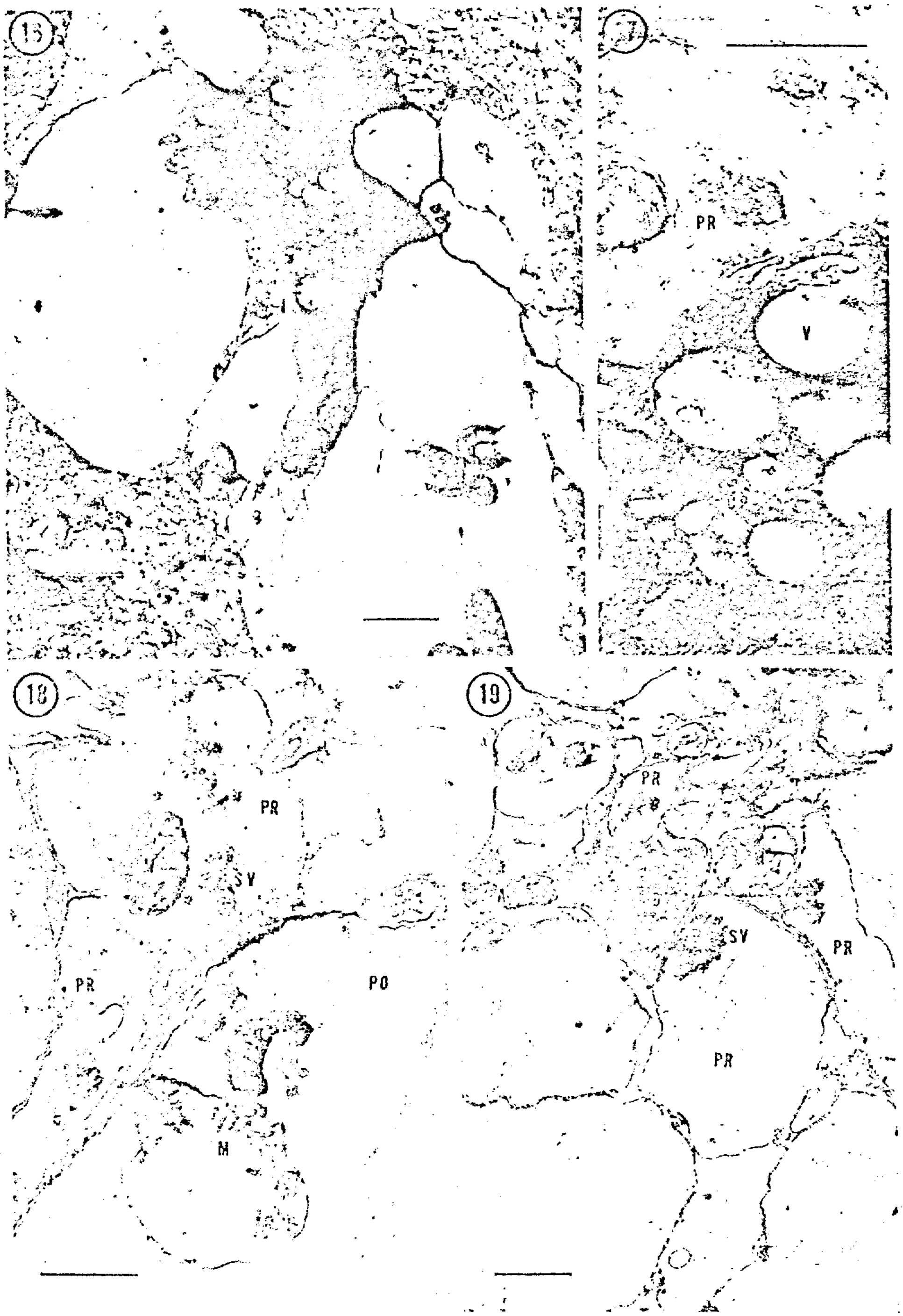


FIGS. 16 to 19.

FIG. 20. Glutamate-injected cortex fixed 6 hours after the administration of the amino acid. The figure shows swollen, electron-transparent pre- and postsynaptic structures. An axonal terminal which does not exhibit signs of swelling (nearly uniform distribution of the synaptic vesicles) synapses with an electron-dense structure, probably a dendritic branch of a shrunken nerve cell. The calibration line indicates 0.5 μ.

Figures 21–24 show ems of glutamate-injected cortices perfused with glutaraldehyde 24 hours after the end of the amino acid administration.

FIG. 21. A swollen postsynaptic structure contacts an axonal ending which is not markedly swollen. Other pre- and postsynaptic structures are present; many have a rather normal appearance. Elements are present containing glycogen granules. One of these seems to synapse with an axonal terminal and thus may be dendritic in nature, others may be glial. The calibration line indicates 1 μ.

FIG. 22. Blood vessel with perivascular glia, which is much less swollen than in Fig. 15, and contains numerous glycogen granules. The calibration line indicates 1 μ.

FIG. 23. A swollen dendritic structure exhibiting microtubules forms a synapse with an axonal terminal which does not show obvious swelling. Calibration 0.5 μ.

FIG. 24. Part of a myelinated fiber containing numerous inclusions, some of which appear to be mitochondria. Calibration 0.5 μ.

Figures 25–28 show glutamate-injected cortices fixed by perfusion with glutaraldehyde 2 days (Figs. 26 and 28) and 3 days (Figs. 25 and 27) after the administration of the amino acid.

FIG. 25. Shrunken electron-dense nerve cell or large dendrite containing vacuoles and mitochondria surrounded by numerous moderately swollen structures which defy identification. Adjacent to this cell is a glia cell with a well-preserved nucleus and cytoplasm which seems slightly swollen. Calibration 5 μ.

FIG. 26. Swollen dendrite synapsing with a normal-appearing presynaptic terminal. Adjacent to this swollen dendrite is an electron-dense structure which may be a process of a shrunken nerve cell. These structures are surrounded by glia elements containing numerous glycogen granules. The calibration line indicates 1 μ.

FIG. 27. A field of nonmyelinated axons which have a normal appearance. Calibration 0.5 μ.

FIG. 28. A glial element which includes, in addition to glycogen granules, debris of shrunken electron-dense nerve cells or dendrites. The calibration line indicates 0.5 μ.

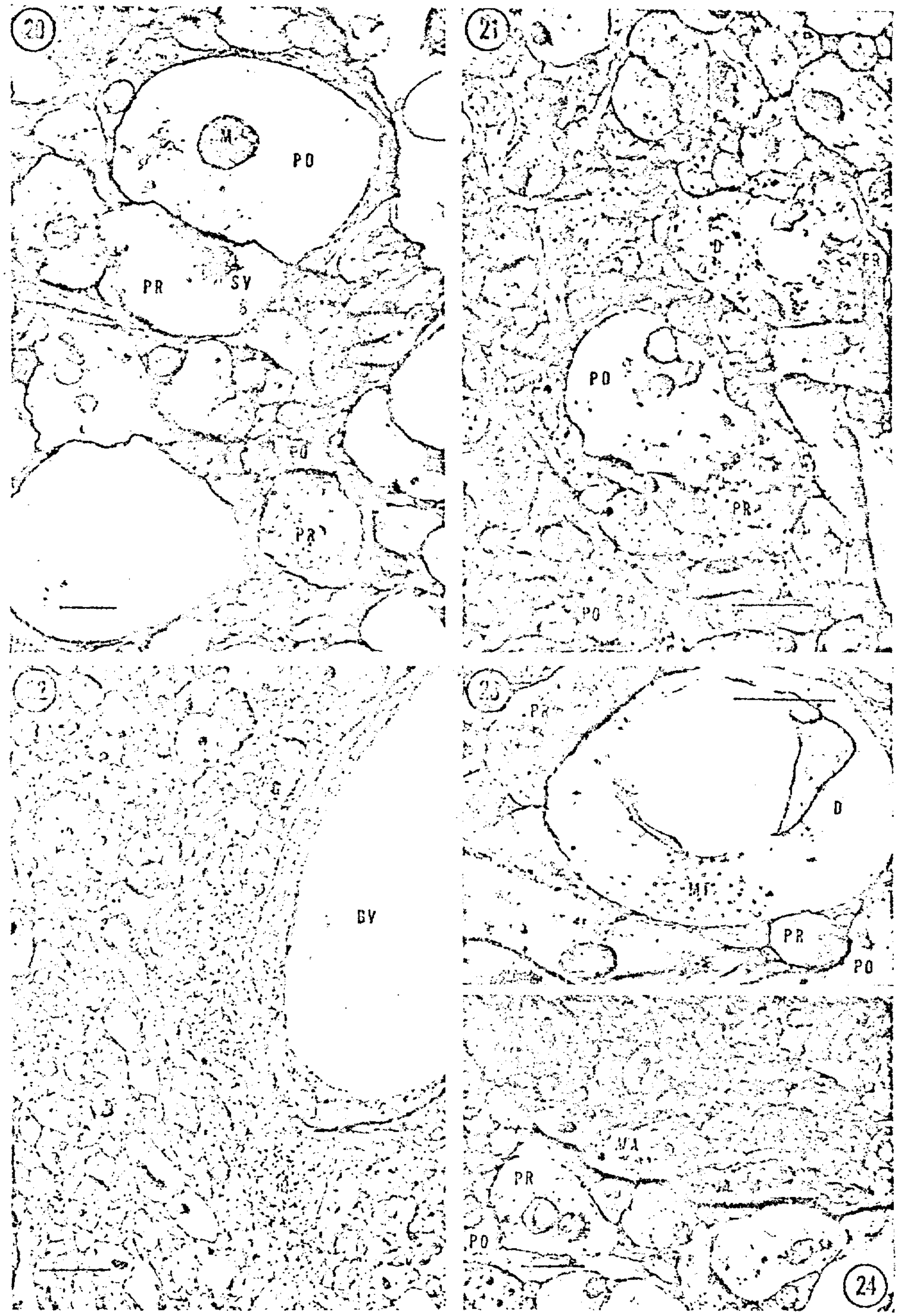
Figures 29–32 show glutamate-injected cortices fixed 1 week after the amino acid administration.

FIG. 29. A number of dendrites with unusually distinct microtubules. Some of the dendrites are probably slightly swollen (nonuniform distribution of the microtubules). Several rather normal-appearing presynaptic terminals are present. Calibration 1 μ.

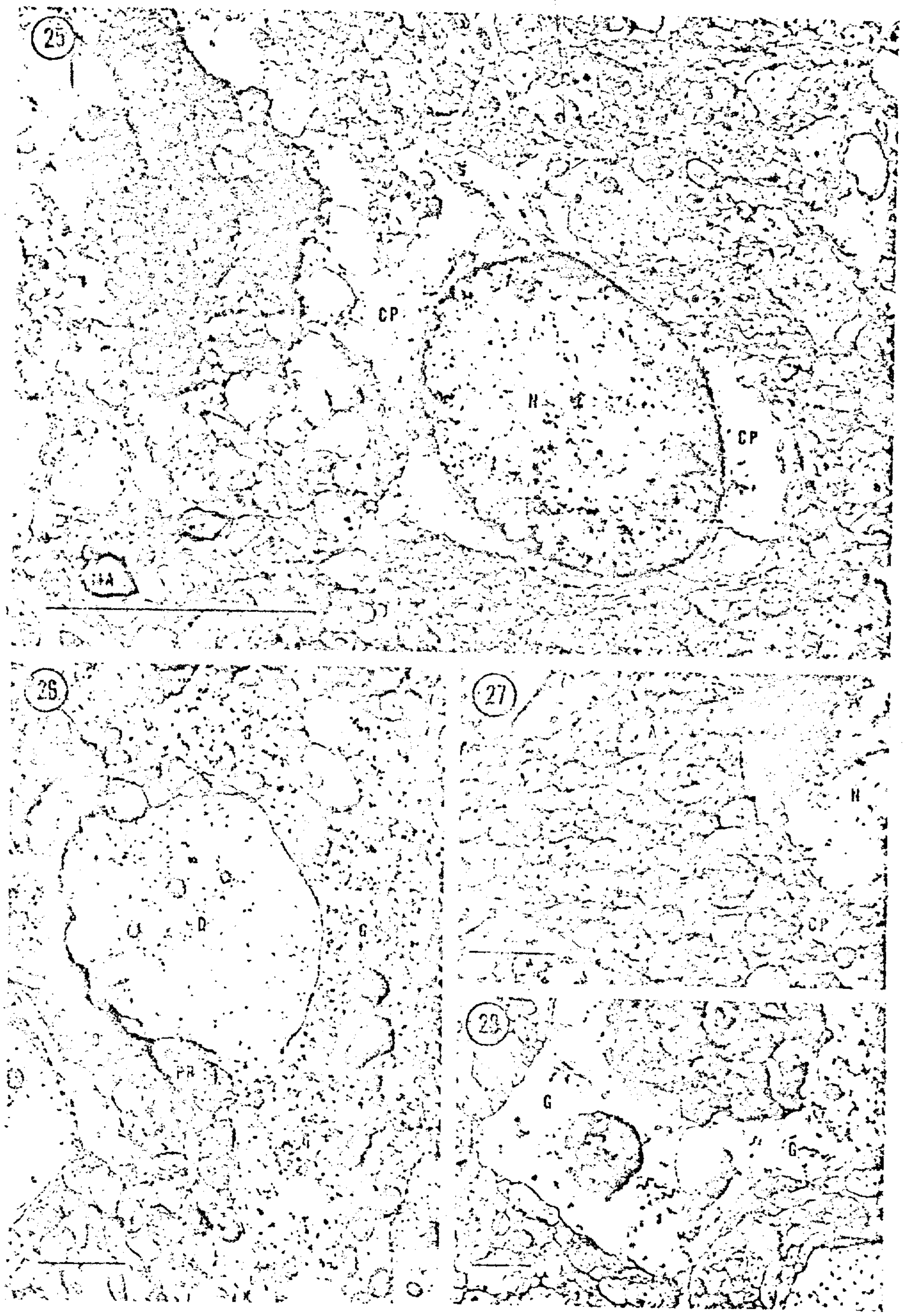
FIG. 30. Capillary with perivascular glia and macrophage exhibiting an endoplasmic reticulum lined with ribosomes. At this stage these cells often contain numerous vacuoles and debris of shrunken nerve cells. The calibration line indicates 5 μ.

FIG. 31. Part of a glia cell is shown with a well-preserved nucleus and cytoplasm which contains ribosomes and vacuoles. The dark material enclosed in the cytoplasm may be residues of nerve cells. There are pre- and postsynaptic structures some of which seem moderately swollen. Calibration 1 μ.

FIG. 32. Many electron-dense structures are present which may be parts of shrunken nerve cells or dendrites. They are often in close contact with glial elements containing glycogen granules. Slightly swollen structures are present but most of the tissue elements have a normal appearance. The calibration line indicates 1 μ.



FIGS. 20 to 24.



FIGS. 25 to 28.

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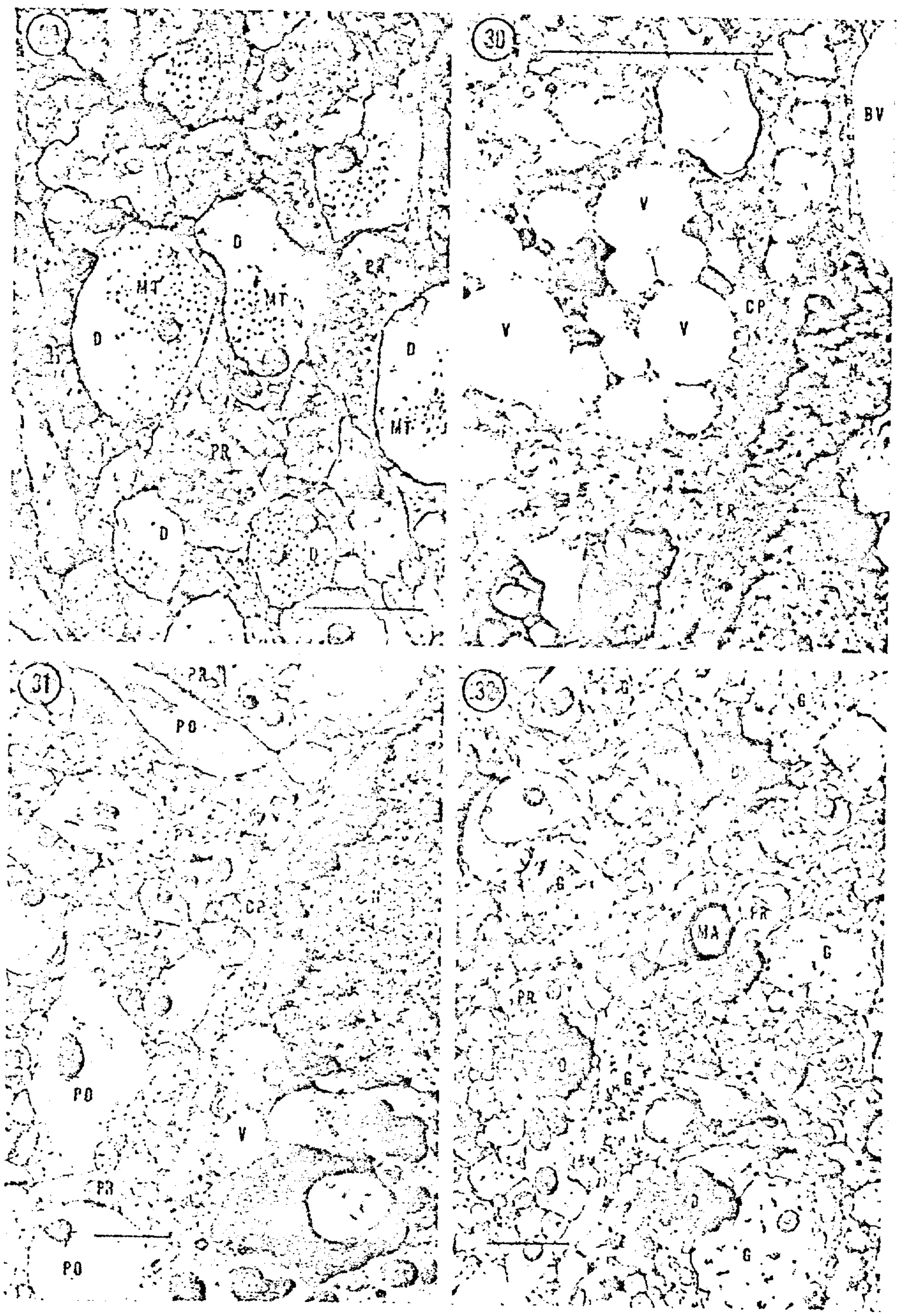

FIGS. 29 to 32.

THE METABOLISM OF GLUTAMIC ACID *

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DURING the past five years my colleagues and I have been studying the metabolism of glutamic acid with particular attention to its relation to the nervous system. Our interest in this subject was stimulated partly by the beneficial effects of giving this amino-acid to certain types of epileptics and mental defectives (Price et al. 1943, Waelsch and Price 1944, Albert et al. 1946). Since most of the diseases of the nervous system have no known counterpart in the lower animals, the biochemist interested in the fundamental mechanisms responsible for the normal and abnormal reactivity of the nervous system has to rely largely on clinical information and is handicapped by the impossibility of experimenting on animals. The lack of methods for an objective assessment of functional changes in the central nervous system presents a further difficulty. Though the clinical observations on the effect of glutamic acid have served to orient our biochemical studies, it is beyond my intention or ability to discuss glutamic acid as a therapeutic agent.

GLUTAMIC ACID IN EPILEPSY

Five years ago (Price et al. 1943) we suggested a study of the effect of *dl*-glutamic-acid hydrochloride in patients with petit mal. This suggestion was made partly because investigations with the aid of the tissue-slice technique had shown that glutamic acid is the only amino-acid capable of taking the place of glucose in maintaining the respiration of brain-cortex slices (Weil-Malherbe 1936). It was also known that an enzyme system which converts glutamic acid into the amide—glutamine (Krebs 1935)—is present in the brain.

Of particular interest was the change in mental status observed in epileptics given glutamic-acid hydrochloride by mouth.

> "Universally mental and physical alertness was increased. The degree of improvement cannot be correlated with the incidence of seizures. . . . Usually the patient is noted to be more energetic and happier, mood swings are less pronounced, behaviour mannerisms are ameliorated, and he is more congenial with associates." (Price et al. 1943.)

Later it was shown that the naturally occurring form of glutamic acid was as effective as the racemic *dl*-glutamic-acid hydrochloride, and it had to be concluded, therefore, that the beneficial effect might be ascribed to that form of glutamic acid which is present in the proteins of the mammalian body and of food (Waelsch and Price 1944). With this form of glutamic acid there was also found the improvement in personality noted before with the *dl*-glutamic-acid hydrochloride. The amount of glutamic acid given to the patients ranged from 9 to 12 g. a day, whereby the daily intake of dietary glutamic acid was increased by about 30–100%.

From clinical reports it appears that some patients with petit mal are benefited by the administration of glutamic acid by mouth. The average incidence of seizures is cut down, but it should be emphasised that the seizures are rarely eliminated completely. Further, it is noteworthy that seizures of the grand-mal type are not affected.

GLUTAMIC ACID IN MENTAL DEFICIENCY

Since it appeared possible that the removal or improvement of an epileptoid mechanism was the reason for the apparent improvement of the mental status, the effect of glutamic acid was tested in mentally retarded persons without any history of convulsive disorder.

In a first preliminary study 10–12 g. of glutamic acid was given by mouth to 8 mental defectives. Its administration was preceded or followed by a period during which a placebo (lactose) was given (Albert et al. 1946). Each person served as his own control; thus the effect of repeated psychometric tests could be assessed. An attempt was made to select mental defectives suspected of a deficiency of the secondary type. The study of 7 of the 8 patients was completed, and it was found that, when glutamic acid was given, the patients' I.Q. score rose 5–17 points (15–35% of the original base level) and dropped to almost the original level when the glutamic acid was discontinued or placebos were substituted (see also Zimmerman et al. 1946, 1947).

These findings warranted a study of the effect of glutamic acid on a larger group of mental defectives, unselected except for the exclusion of all who had a history of convulsive disorders. Such a group was made available to us by the Bureau for Retarded Children, Board of Education of the City of New York. Table I gives the preliminary results of this study, which is still being continued. The I.Q. score of 30% of the children on glutamic acid rose 5 points or more, whereas none of the children on placebos showed a comparable gain in mental performance. Of 5 children who benefited from the administration of glutamic acid 3 regressed back to the original I.Q. level during a subsequent placebo period. The psychologist who tested the mental performance was not informed whether the children were receiving glutamic acid or placebos.

MODE OF ACTION ON BRAIN

The effects of glutamic acid in epileptics and in mental defectives were observed after the administration of glutamic acid by mouth. Mayer-Gross and Walker (1947) have shown that glutamic acid given intravenously to patients in insulin coma restores consciousness at a considerably lower blood-sugar level than when glucose is used. This effect, however, is not specific for glutamic acid, since it may be obtained with other amino-acids (W. Mayer-Gross, personal communication). At present there is no experimental evidence to indicate a direct effect of glutamic acid on the brain in the intact organism. Though the administration of glutamic acid by mouth produces an increase of its concentration in the blood (see below) it may affect the brain indirectly by an effect on the metabolism of some other organ. It should be emphasised that in all our experiments the free amino-acid was given, and the question arises whether the same effect would be obtained from the same amount of glutamic acid bound in a protein.

On the present experimental evidence we cannot state that the effect of glutamic acid is specific. Many other substances metabolically related or unrelated to it may well have similar effects. On the other hand, there are few metabolites in carbohydrate and amino-acid metabolism which are not linked by some biochemical mechanism with glutamic acid, so it appears justified to concentrate at present on the study of glutamic acid.

If we assume that glutamic acid exerts its effect after its absorption through the intestinal wall and not by modifying the absorption of other compounds or by influencing the intestinal flora, and if we assume further that its calorigenic action is not responsible, we are faced with an interesting biochemical problem. Glutamic acid is one of the amino-acids which can be synthesised in the body; and only under very special conditions, when glutamic acid and all of its metabolic sources, such as ornithine and proline, are excluded, does the absence of glutamic acid affect the growth of rats (Womack and Rose 1947). Such is surely not the

* Based on a lecture presented at the Institute of Psychiatry, Maudsley Hospital, London, on June 15, 1948. The studies reported here were supported by grants from the Rockefeller Foundation, the New York Foundation, and the Williams-Waterman Fund of the Research Corporation.

TABLE I—EFFECT OF GLUTAMIC ACID AND PLACEBOS ON MENTALLY RETARDED PERSONS (I.Q. RANGE 18–58; C.A. RANGE 6–17)

First period (4 months)	Glutamic acid	Placebo
No. of persons	20	27
No. of persons showing increase of 5 points or more	9	0
No. of persons showing decrease of 5 points or more	0	1
Second period (4 months)	**Placebo**	**Glutamic acid**
No. of persons	5	16
No. of persons showing increase of 5 points or more ..	0	2
No. of persons showing decrease of 5 points or more	3	0

situation in those patients whose mental performance is benefited by the administration of glutamic acid, since, in addition to the amounts available from synthesis in the body, they are receiving a large quantity, probably a multiple of the administered dose, in the protein of the food. It therefore seems that, in this instance, the addition of a body component which is supplied in large amounts by the diet and is also synthesised in the body may exert a beneficial effect if added in relatively small amounts to the diet.

A study of the metabolism of glutamic acid cannot be dissociated from that of its amide, glutamine, which occurs in considerable concentration in proteins and in the non-protein fraction of plant and animal tissues. There are enzyme systems in mammalian tissue which convert glutamic acid into the amide or split the amide to glutamic acid and ammonia (Krebs 1935). The importance of glutamine has been emphasised by the finding that it is an essential nutrient for certain micro-organisms, and in this respect its place cannot be taken by glutamic acid (McIlwain et al. 1939).

ABSORPTION AND DISTRIBUTION

As a first step in the study of the utilisation and metabolism of glutamic acid and glutamine, their absorption and distribution in the tissues were studied. Before reporting this aspect of our study, I wish to discuss the concentrations of glutamic acid and glutamine in the blood-plasma of epileptic and non-epileptic persons (table II; for methods of determination see Prescott and Waelsch 1946, 1947).

For comparative purposes the concentration of an essential amino-acid, phenylalanine, was determined. Neither in the absolute values, nor when the amount of the analysed amino-acids was expressed as a percentage of the total amino-acids, was any difference found between normal persons and epileptics. One point deserves particular attention—the ratio between glutamine and glutamic acid. The amount of glutamine in plasma was about ten times that of glutamic acid, and similar ratios were found in ox, cat, and rat blood. This finding may be of particular significance, since the amide does not take part in many of the metabolic reactions in which glutamic acid is a specific substrate (see below). On the other hand, a varying but large part of glutamic acid in proteins is present as glutamine, and therefore different ratios of the two compounds are offered in the dietary proteins.

The question arose whether any interconversion occurs between glutamic acid and glutamine during their absorption through the intestinal wall (Bessman et al. 1948). Glutamic acid or glutamine was introduced into the small intestine of cats, and the plasma of the portal blood was analysed for its content of glutamic acid and glutamine, 15 and 30 minutes after the introduction of the two compounds (table III). Glutamic acid and glutamine were absorbed independently of each other since immediately after the introduction of either compound there was a sharp rise in the plasma concentration of the administered substance only. When glutamic acid was given, there was a drop in glutamine concentration after 15 minutes, followed by a rise above the baseline after 30 minutes. This decrease in the 15-minute values is interpreted as a shift of glutamine from the plasma into the tissues.

A similar decrease has been found also in peripheral blood after giving glutamic acid by mouth to man, and this appears to be part of a general phenomenon, since

TABLE III—CONCENTRATION OF FREE GLUTAMIC ACID (ACID) AND GLUTAMINE (AMIDE) IN PLASMA OF PORTAL VEIN (CAT) AFTER INTRA-INTESTINAL ADMINISTRATION OF 100 MG. OF GLUTAMIC ACID OR OF GLUTAMINE

Values expressed as mg. per 100 ml. of plasma *

—	Glutamic acid given				Glutamine given			
Cat no.	1		2		3		4	
Min. after administration	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0	2·8	10·7	1·7	10·4	2·5	6·4	1·6	5·9
15	12·9	4·2	6·6	8·3	3·9	12·7	1·9	12·3
30	14·8	13·0	1·0	15·5	3·1	25·8	1·6	8·8

* Per cent. blood-cells in cat no. 1 at 0, 15, 30 minutes: 44, 36, 35. In cats no. 2–4: change in blood-cell volume smaller than 3%.

an increased concentration of glutamic acid is accompanied by a fall in the levels of glycine and residual amino-acid nitrogen (Christensen et al. 1948).

The significance of this finding is still obscure. The increase of the glutamine concentrations at 30 minutes over the values at 0 time is interpreted as the mobilisation of tissue glutamine or the release of glutamine formed in the organs from the administered glutamic acid. We may assume that glutamic acid and glutamine are liberated from the dietary proteins by the enzymes of the digestive tract without extensive interconversions, and our experiments show that the amide and its parent amino-acid may be absorbed without significant changes. This may mean that the administration of 10–15 g. of glutamic acid by mouth, a small amount compared with the potential intake of glutamic acid (glutamic acid plus glutamine), may increase the *relative* amount of glutamic acid considerably. Administration of an additional 10 g. of glutamic acid with 100 g. of food

TABLE II—FREE GLUTAMIC ACID, GLUTAMINE, PHENYLALANINE, AND TOTAL AMINO-NITROGEN IN PROTEIN-FREE FILTRATES OF HUMAN PLASMA

—	Glutamic acid		Glutamine		Phenylalanine	Amino-nitrogen
	NE	E	NE	E		
No. of samples ..	13	19	12	18	17	17
Range (mg. per 100 ml.) ..	0·4–1·7	0·3–1·2	4·5–10·6	4·8–10·1	0·6–1·3	3·4–5·2
Mean (mg. per 100 ml.) ..	0·7	0·7	8·0	7·8	0·95	4·2
Standard error	± 0·07	± 0·05	± 0·5	± 0·4	± 0·06	± 0·1

NE, non-epileptics; E, epileptics.

TABLE IV—CONCENTRATION OF FREE GLUTAMIC ACID (ACID) AND GLUTAMINE (AMIDE) IN PLASMA OF PERIPHERAL BLOOD AFTER ADMINISTRATION OF GLUTAMIC ACID BY MOUTH TO HUMAN SUBJECTS (1 G. PER 10 KG. OF BODY-WEIGHT)

Values expressed as mg. per 100 ml. of plasma

Hours after administration	Subject 1		Subject 2		Subject 3		Subject 4	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0	0·6	8·4	0·6	10·6	1·2	8·4	0·8	10·3
1	1·0	10·3	1·2	14·0	5·0	5·8	9·5	8·3
2	1·0	8·9	0·8	11·3	0·7	6·3	1·0	10·0

protein, containing 10 g. of glutamine and 10 g. of glutamic acid, increases the intake of potential glutamic acid from 20 to 30 g. but doubles the intake of the amino-acid itself.

LEVELS IN BLOOD AND TISSUES

As the next step in tracing the pathway of glutamic acid, the changes in the peripheral blood were determined after the administration of glutamic acid by mouth (Bessman et al. 1948). Glutamic acid 1 g. per 10 kg. of body-weight was given by mouth to four human subjects, and the concentrations of glutamic acid and glutamine in the plasma of the peripheral blood were determined 1 and 2 hours afterwards. The amounts given corresponded to two or three times the dose given to epileptics or to mental defectives.

In all four experiments (table IV) the administration of glutamic acid led to an increase in the glutamic-acid content of the peripheral blood, but the extent of the increase varied considerably. In those experiments in which only a small rise in the glutamic-acid level was found a concomitant significant increase of glutamine was observed; but where the glutamic-acid level rose considerably the glutamine values decreased at first and increased later. Here the picture was very similar to that found in the portal blood of cats after the intra-intestinal administration of glutamic acid. The fall in the plasma-glutamine level, which appears to be accompanied by a fall in the levels of other amino-acids, may be a clue to the effect of giving glutamic acid. These findings indicate the discouraging complexity of the changes resulting from the administration of glutamic acid.

TABLE V—TISSUE DISTRIBUTION OF GLUTAMIC ACID (ACID) AND GLUTAMINE (AMIDE) AFTER INTRAVENOUS INJECTION IN MICE (1·3 MG. PER G. OF BODY-WEIGHT)

Values expressed in mg. per 100 g.

Min. after administration	Glutamic acid given		Glutamine given	
	Acid	Amide	Acid	Amide
		Brain		
0	145	53	145	53
15	153	80	140	116
		Liver		
0	19	35	19	35
15	70	32	300	90

The administration of glutamic acid by mouth may increase both the glutamic acid and glutamine concentrations in the peripheral blood, thereby fulfilling a condition obligatory for an effect of glutamic acid on the brain. A direct influence of glutamic acid or of glutamine on the metabolism of any organ can only be expected after the entry of these compounds into the organ.

The uptake of glutamic acid and its amide by tissues was studied by tissue analyses after the intravenous administration of the two compounds. It should be kept in mind that the intravenous injections of large amounts of a single amino-acid create unphysiological conditions. Different results may be obtained when the amino-acid and its amide are accompanied by all the other protein components, as under physiological conditions. The uptake of glutamic acid and glutamine by several tissues during the first 30 minutes after intravenous administration to mice and rats was determined. Preparatory to this study the normal concentrations of glutamic acid and its amide in several tissues were estimated. Though the glutamine values for the protein-free filtrates of tissues of the dog and of blood-plasma of several species have been reported (Archibald 1945), no information on the concentration of glutamic acid was available.

Our analyses (tables V and VI, values at 0 time) show that some organs (muscle and liver), of the rat and mouse contain an excess of glutamine over glutamic acid, whereas in others (brain and kidney) the concentration of glutamic acid exceeds that of glutamine. In the brains of mice and rats as well as in those of rabbits, calves, and pigeons a twofold to threefold excess of the amino-acid over its amide was found, and the absolute concentration of glutamic acid was about twice that of glucose.

TABLE VI—TISSUE DISTRIBUTION OF GLUTAMIC ACID (ACID) AND GLUTAMINE (AMIDE) AFTER INTRAVENOUS INJECTION IN RATS (1·3 MG. PER G. OF BODY-WEIGHT)

Values expressed in mg. per 100 g.

Min. after administration	Glutamic acid given		Glutamine given	
	Acid	Amide	Acid	Amide
		Brain		
0	133	62	133	62
10	120	65	120	68
20	140	56	140	84
		Liver		
0	33	55	33	55
10	94	38 –	210	210
20	72	45 –	200	73
		Muscle		
0	21	32	21	32
10	40	35	18	68
20	18	42	13	53
		Kidney		
0	98	19	..	..
10	400	29	..	..
20	424	39	..	..

It would be feasible to consider glutamic acid as a respiratory substrate of brain tissue, particularly since a determination of the respiratory quotient by analysis of the arterial-venous difference cannot differentiate between the utilisation of glucose and that of glutamic acid. Recent investigations on brain respiration in progressive hypoglycæmia have led to the conclusion that under such conditions the brain utilises its own carbohydrate stores or some other endogenous substrate (Kety et al. 1948).

ORIGIN OF GLUTAMIC ACID IN THE BRAIN

The high concentration of glutamic acid in brain made it of particular interest to ascertain whether the amino-acid originates from the circulating blood. After the intravenous administration of glutamic acid to mice and rats no significant increase was found in the concentration of glutamic acid in the brain; the length of the experimental period was such that metabolic conversions would probably not significantly distort the changes resulting from uptake into the tissue (tables V and VI). Whereas liver and muscle took up considerable amounts of glutamic acid, the amount of glutamic acid in the brain varied within the range found in the control animals. This led to the conclusion that glutamic acid as such apparently does not enter the brain.

Such a result had been suggested before by studies in which the total amino-acid nitrogen of the brain was measured after the administration of glutamic acid (Friedberg and Greenberg 1947), but only the specific determination of glutamic acid can give an unequivocal answer. A considerable increase in the concentration of glutamic acid occurred in the liver of mice, and in the liver, muscle, and kidney of rats. No indication of conversion of glutamic acid to glutamine was found except in the rat kidney. There may have been a slight increase in the glutamine content of the mouse brain.

After the injection of glutamine there was a considerable increase of that substance in the mouse brain and a slight increase in the rat brain. An enormous increase in the amount of glutamine in the liver was observed, and an immediate liberation of glutamic acid from the accumulated glutamine. The increase in muscle-glutamine was comparable to the increase in glutamic acid observed after the administration of the amino-acid. The results of the injection experiments suggest that glutamine enters the liver, and possibly also the brain, with greater ease than does glutamic acid.

Glutamic acid does not enter the brain of animals under the particular conditions of our experiments. It cannot be concluded that the same holds true for the human brain or in pathological conditions, such as epilepsy and mental deficiency. We cannot state definitely whether the glutamine of the blood is likewise excluded from the brain, since in some of the experiments an increase in the amount of "apparent glutamine" (Prescott and Waelsch 1947) in the brain tissue was noted.

Though glutamic acid and glutamine are possibly derived from the glutamine of the circulating blood, it appeared desirable to find out whether the two amino-acids are synthesised in the brain itself. In preliminary experiments slices of brain cortex from a guineapig were shaken in Krebs-Ringer-phosphate solution under aerobic conditions, and the concentrations of glutamic acid and glutamine were determined in the absence and presence of glucose after different intervals of time. In the absence of glucose there was a rapid decrease in the concentrations of glutamic acid and glutamine in the slices. If glucose was added to the exhausted slices, the content of glutamic acid and glutamine was increased. If glucose was added at the beginning, the depletion of the slices in glutamic acid and glutamine was arrested. These results would indicate that glutamic acid and glutamine are synthesised in the brain slices, were in not for the possibility that the increase in the amount of glutamic acid is the result of autolytic processes by which glutamic acid might be liberated from protein.

The experimental evidence presented so far is compatible with the view that the effect of glutamic-acid administration on the metabolism and function of the central nervous system does not result from a direct action of the amino-acid on the brain. It is doubtful whether glutamine can pass the blood-brain barrier and thereby supply the brain with glutamine and glutamic acid. The alternative is that the brain manufactures the amino-acid and the amide, and that the effect of administered glutamic acid on the brain is indirect through other organs. The finding that an increased glutamic-acid level in the blood leads to a removal of other amino-acids adds another complicating factor, and we cannot exclude the possibility that the effect of glutamic-acid administration is the result of the concentration of substances in the tissues by such a mechanism.

GENERAL CONSIDERATIONS

Glutamine appears to enter the tissues more easily than does the parent amino-acid. The preferential entrance of the amide into the tissues may have considerable significance for the organism. Glutamic acid is metabolically one of the most active of amino-acids. It mediates the entrance of ammonia into the amino-acid pool and it takes part in transamination reactions. It is also the parent substance of ketoglutaric acid, one of the essential members of the tricarboxylic-acid, or Krebs, cycle by which pyruvic acid originating from carbohydrate breakdown is finally burned to carbon dioxide and water. This part of the metabolism of carbohydrates contributes the lion's share of energy during their metabolic degradation. It is assumed today that the tricarboxylic-acid cycle is also the pathway for the oxidative breakdown of fatty acids and the carbon skeleton of many amino-acids. Whereas glutamic acid, or ketoglutaric acid, participates in all these processes, as well as in the synthesis of urea (Cohen and Grisolia 1948) and in the reactivation of the enzyme system synthesising acetylcholine (Nachmansohn et al. 1943), glutamine is inert in all these metabolic reactions. It is a depot from which glutamic acid is easily liberated by enzymatic action but which by itself does not change the rate of the basic metabolic reactions proceeding in the tissue (cf. Chibnall 1939).

This concept of the function of glutamine disregards for the moment other functions of the amide, of which we know very little—e.g., the removal of ammonia, and its rôle in glycolysis and the synthesis of protein.

The concept that glutamine represents a store of metabolically inert glutamic acid and ketoglutaric acid, easily mobilised by enzymatic action—similar to the rôle of glycogen in carbohydrate metabolism—may be extended to explain the presence of a large concentration of free glutamic acid and of glutamine in brain and other organs. The rate of the tricarboxylic-acid cycle determines the amount of energy derived from the combustion of carbohydrates, and a certain, if only catalytic, concentration of each of its components is needed to carry the cycle at a rate compatible with average physiological function.

The physiological rhythm of function and inactivity of an organ may find one of its metabolic expressions in the rate of the tricarboxylic-acid cycle, particularly in organs such as the brain, which derives its energy requirements from the breakdown of glucose. The concentration of the different components fed into the tricarboxylic-acid cycle from many metabolic pathways undoubtedly varies widely and requires some physiological regulation. It is likely that such a basic metabolic cycle will be well buffered to ensure a physiological rate. One of the metabolic buffers of the tricarboxylic-acid cycle may be the system: glutamic acid, glutamine. The presence of glutamine and of glutamic acid in large concentrations may doubly ensure the adequate supply of ketoglutaric acid. It is too early to speculate whether the large amounts of glutamic acid and the smaller amounts of glutamine in brain are the metabolic expressions of its functional state, which has to be kept within carefully guarded limits.

We are still a long way from understanding the effect of glutamic acid on the central nervous system. By trying to reach an understanding of this effect, we may hope to learn something about the metabolism of amino-acids and its relation to the function of the nervous system.

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2. GLUTAMIC ACID AND ITS RELATION TO THE NERVOUS SYSTEM

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The functions of glutamic acid in the central nervous system are not only of great intrinsic interest, but they have recently become the focal point of much publicity owing to the clinical and psychological effects which glutamic acid is alleged to produce. Some aspects of the problem have been considered in a recent review (Weil-Malherbe, 1950); the present discussion will therefore be mainly concerned with the literature published in the two years which have elapsed since the above review was written.

THE CONCENTRATION OF FREE GLUTAMIC ACID AND RELATED SUBSTANCES IN BRAIN

The nitrogen content of 30 nitrogenous components of deproteinized brain extracts (mostly of the rat), for which quantitative data are recorded in the literature, accounts for 80–90% of the total non-protein nitrogen of rat brain. In the histogram of Fig. 1 these substances have been arranged in descending order on an arbitrary molar scale. Glutamic acid heads the list, followed, in third and fourth place, by the closely related compounds, glutamine and γ-aminobutyric acid. If only the free amino-acids of brain are considered, the molar concentration of glutamic acid accounts for about 30%, and that of the three compounds, glutamic acid, glutamine and γ-aminobutyric acid, together, for about 70% of the total.

Compared with the majority of free amino-acids in brain, the concentration of glutamic acid is disproportionately high. Its special position indicates a special function; moreover, it indicates a stoichiometric, rather than a catalytic function.

THE ENZYMIC TRANSFORMATIONS OF GLUTAMIC ACID IN BRAIN AND THEIR PHYSIOLOGICAL FUNCTIONS

Brain contains enzyme systems for the oxidation, transamination, amidation and decarboxylation of glutamic acid (for the literature prior to 1949 see Weil-Malherbe, 1950). In its ability to undergo oxidation, amidation and decarboxylation in brain glutamic acid is unique amongst amino-acids, and it also has a central function in transamination reactions. As far as the transamination reactions and the oxidation of glutamic acid are concerned, brain resembles

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other tissues, such as muscle, but it differs from most of them in containing amidation and decarboxylation systems.

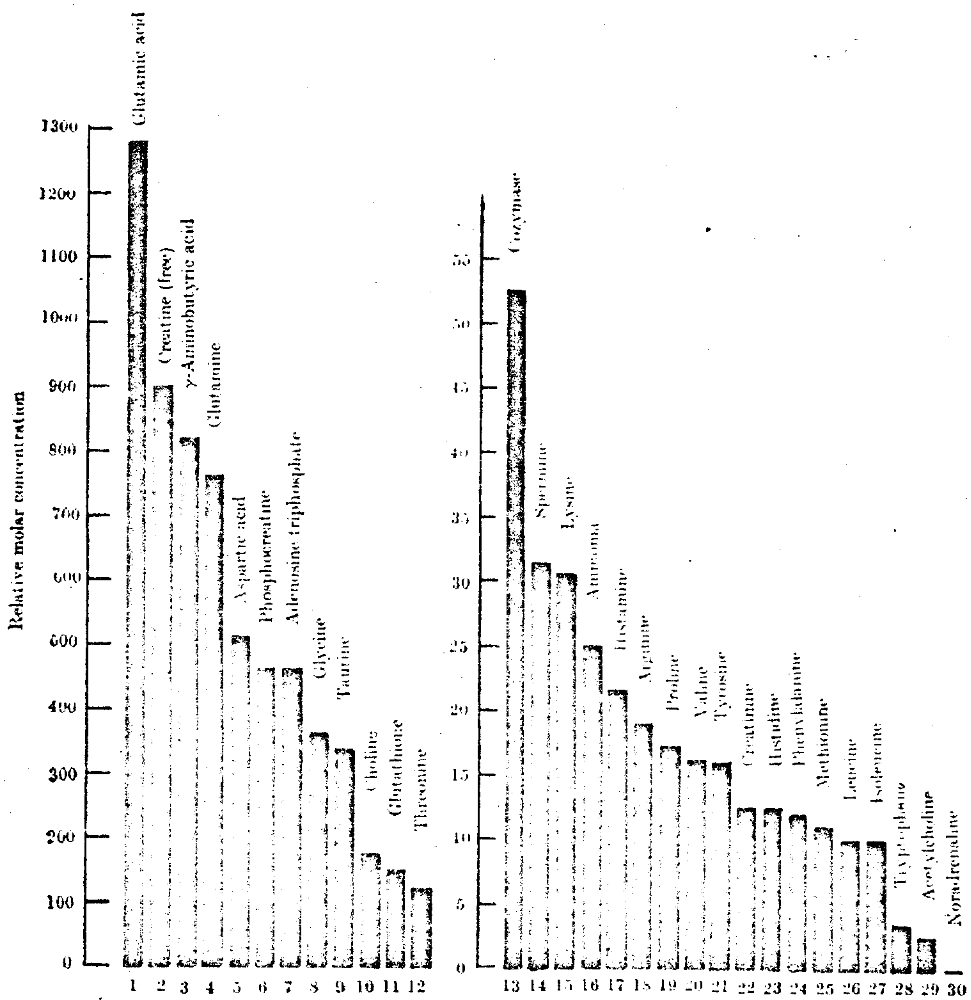

Fig. 1. Relative molar concentrations of nitrogenous components of deproteinized brain extract.

A clue to the functions of these enzyme systems may perhaps be obtained from a consideration of those special properties of glutamic dehydrogenase which set it apart from other amino-acid oxidases. Glutamic dehydrogenase, in common with other nicotinamide enzymes, has a relatively low oxidation-reduction potential. Accordingly, the reaction :

$$\text{glutamic acid} + \text{Co.I} \underset{}{\overset{\text{dehydrogenase}}{\rightleftharpoons}} \text{iminoglutaric acid} + \text{CoH}_2\text{.I}$$

is easily reversible: the equilibrium lies in fact far to the left, in favour of glutamic acid synthesis. This equilibrium is, however, only attainable if the secondary non-enzymic hydrolysis of iminoglutaric acid to ketoglutaric acid and ammonia, according to the equation

$$\text{Iminoglutaric acid} + H_2O \rightleftarrows \alpha\text{-ketoglutaric acid} + NH_3,$$

is prevented. Iminoglutaric acid is therefore only stable in the presence of ammonium ions. In the respiring cell where the concentration of citric acid-cycle intermediates has reached a steady state, the appearance of NH_4^+ will automatically lead to the synthesis of glutamic acid. The reductive amination of ketoglutarate which takes place in slices or homogenates of liver on addition of NH_4^+ so depletes the level of C_4-acids that the metabolism of pyruvate is largely switched to acetoacetate production (Recknagel & Potter, 1951). The presence of glutamic dehydrogenase thus prevents or counteracts the accumulation of NH_4^+ in the cell.

It is known that the ammonia content of nervous tissues increases during periods of increased nervous activity and *vice versa*. Dawson (1951) has shown that the concentration of both glutamic acid and ammonia in the brains of rats killed during thiopentone anaesthesia is reduced, especially when the drop in body temperature is not prevented. In rats whose body temperature was maintained at 37° during anaesthesia the reduction was smaller. It seems reasonable to connect the decreased concentration of glutamic acid with the reduction in ammonia formation consequent upon the lowering of functional activity in the brain.

The amidation system easily fits into the picture. When brain slices respire in a glucose-free medium, a steady formation of ammonia is observed, while there is little change in the concentration of amide N. In a glucose containing medium, on the other hand, hardly any free ammonia is formed, although there is a considerable increase of non-protein N, part of which appears as amide-N (Weil-Malherbe, 1951). This observation suggests the existence of an ammonia-binding mechanism leading from ketoglutarate to glutamate, and from glutamate to glutamine, a substance generally accepted as the transport form of ammonia in the body.

The transamination reactions, too, may take part in the process of ammonia disposal. It is probably no coincidence that the three keto-acids involved in the most active transamination systems, pyruvate, oxaloacetate and ketoglutarate, are members of the citric acid cycle. This limitation suggests that the cell may exploit the constant availability of pyruvate and oxaloacetate and use them as acceptors of amino-groups from glutamate whenever the concentration of ketoglutarate is insufficient to cope with the influx of ammonia, or would thereby be reduced to a dangerously low level.

Important as the ammonia-binding function of the enzymes grouped round glutamic acid undoubtedly is, it is not suggested that it is the only one. It is, for instance, now becoming apparent that the scope of the transamination mechanism is less restricted than was supposed for some time (Cammarata & Cohen, 1950; Hird & Rowsell, 1950; Rowsell, 1951). Unfortunately no studies have yet been made with brain tissue, but if the situation is similar to that in liver, kidney or heart, glutamic acid might be involved in the synthesis of a large number of amino-acids. It has also been found that, in liver, the amide group of glutamine can be used for the amination of certain keto-acids (Meister & Tice, 1950). Here again, it is not known if similar reactions occur in brain.

According to a recent theory the synthesis of glutamine, a process coupled with adenosine triphosphate breakdown, is the mechanism by which phosphate bond energy is fed into peptide bond synthesis. Glutamine is assumed to exchange its amide group for the α-amino-group of an amino-acid or peptide in a process termed transpeptidation. Proteinases have been shown to bring about the linkage of amino-acid amides in this way (Fruton, Johnston & Fried, 1951) and a reaction has been described in sheep kidney extract, in which the cysteinylglycine residue of glutathione is replaced by another amino-acid resulting in the formation of a new γ-glutamyl peptide (Hanes, Hird & Isherwood, 1950).

That similar reactions may occur in brain is suggested by the presence of an enzyme (or enzymes) which catalyses the exchange of the glutamine amide group with ammonia or hydroxylamine (Waelsch, 1951 *a*; Schou, Grossowicz, Lajtha & Waelsch, 1951). Whether these reactions have a wider significance as models of peptide bond synthesis remains to be seen.

The occurrence of glutamic decarboxylase in brain has been discovered independently by Roberts & Frankel (1951 *a*, *b*) and Wingo & Awapara (1950). The product of its action, γ-aminobutyric acid, is present in brain in remarkably high concentration (Roberts & Frankel, 1950; Awapara, Landua, Fuerst & Seale, 1950) and the enzyme itself is 20 times more concentrated in brain than in liver or kidney (Roberts & Frankel, 1951 *b*). These facts are sufficient to attribute to glutamic decarboxylase a function of major importance in brain metabolism. The enzyme requires pyridoxal-5′-phosphate as coenzyme (Roberts & Frankel, 1951 *b*; Roberts, Younger & Frankel, 1951; Viscontini, Ebnöther & Karrer, 1951) and a decrease of its activity may be one of the factors responsible for the neurological and psychiatric manifestations of pyridoxine deficiency (Wintrobe, Miller, Follis, Stein, Mushatt & Humphreys, 1942; Follis & Wintrobe, 1945; Hawkins & Barsky, 1948; Davenport & Davenport, 1948).

As to the possible function of glutamic decarboxylase, a connection with the ammonia-binding mechanism is not apparent. Provided the

CO_2 evolved is removed from the cell, a decrease of acidity and a liberation of fixed base will result. Such a mechanism may be called upon in sudden bursts of glycolysis in order to reinforce or restore the buffering capacity of the cell. It is known that the formation of bacterial decarboxylases, a group of adaptive enzymes, is much increased when the cells are grown in an acid or poorly buffered medium (Gunsalus, 1950). This lends support to the assumption that protection from acidity is a function of these enzymes.

Binkley (1951) has expressed the view that the enzymes concerned with the hydrolysis of glutamine and glutathione constitute an ion exchange system which functions in the maintenance of the ionic environment of the cell.

The effect of glutamic acid on the restoration and maintenance of the intracellular potassium content of respiring brain slices has probably a simpler explanation, since approximately equivalent quantities of glutamate and K^+ move into the cells together (Terner, Eggleston & Krebs, 1950). It seems that in this process K^+ is merely the preferential cationic complement of the glutamate anion which is itself absorbed by brain cells from the surrounding medium with great avidity (Stern, Eggleston, Hems & Krebs, 1949). The absorption of glutamate seems simultaneously to facilitate the absorption of other amino-acids: glutamic acid alone out of 17 amino-acids, when fed to guinea pigs, led to an increase of the ratio of intracellular to extracellular glycine and other amino-acids in liver and muscle (Christensen, Streicher & Elbinger, 1948).

GLUTAMATE OXIDATION AND THE SUPPLY OF ENERGY

The synthesis of glutamine from ammonium glutamate and the absorption of glutamate and potassium ions by brain slices are dependent on the presence of glucose as a source of energy. It follows that the cell cannot derive the energy required for these activities from the oxidation of glutamate itself. This tallies well with the experiments of McIlwain (1951) who showed that glutamic acid fails to restore, or even maintain, the phosphocreatine concentration of brain slices (see however Klein, 1945) or to duplicate the effect of glucose on the response of brain slices to electrical stimulation. The comparatively small decrease of glutamic acid concentration in the brains of hypoglycaemic rats (Dawson, 1950) does not necessarily indicate the utilization of glutamic acid as an emergency fuel, but may be the result of a change in the relative rates of synthesis from ketoglutaric acid and utilization in amidation, transamination or decarboxylation reactions. It is likely, therefore, that glutamic acid does not normally act as an energy-supplying fuel and that its oxidizability is merely incidental to its other functions in brain.

Although this view has already been clearly stated in 1936 (Weil-Malherbe, 1936), the conception of glutamic acid as a fuel in brain metabolism has gained currency and has often been advanced as the rationale for glutamic acid therapy in mental deficiency and in epileptic disorders of the petit mal type.

GLUTAMIC ACID THERAPY OF MENTAL DEFICIENCY AND EPILEPTIC DISORDERS

In the last two years reports on the glutamic acid treatment of mental deficiency have appeared from several centres. The work of Zimmerman and his collaborators has been criticized on the grounds that their controls were inadequate: objections were also raised to their use of statistics (Ellson, Fuller & Urmston, 1950). Several investigators failed to find any significant difference between the test scores of the treated and control groups (Ellson *et al.*, 1950; Kerr & Szurek, 1950; Loeb & Tuddenham, 1950; McCulloch, 1950; Quinn & Durling, 1950; Milliken & Standen, 1951). Zimmerman & Burgemeister (1951), however, pointed out that many of their critics did not, in fact, repeat their experiment since they used a solution of sodium glutamate instead of giving glutamic acid in powder form. The difference may seem trivial, but Pond & Pond (1951) have demonstrated, in experiments on petit mal cases, that the two procedures give different results, not only in their clinical effects, but also in the rate of absorption and excretion of glutamate. the alkali reserve and the chloride concentration of plasma was unaffected.

The results of the Zimmerman group have been confirmed by the following authors: Harney (1950), Bessman (1950), De la Fuente Muniz, Zuniga & Zanowsky (1950), Schwöbel (1950), Delay & Pichot (1951), Schaechter (1951) and Contini-Poli (1950). Several of these papers were accessible to the reviewer only in abstracts, but it appears that only the last-named author carried out simultaneous psychological tests on a comparable number of control cases. A large scale study on the effect of glutamic acid rather than sodium glutamate, including the simultaneous study of a well-matched control group without the psychologist knowing to which group the subject he is testing belongs—has still to be carried out.

In such an investigation another point would have to receive more attention than hitherto, *viz.* the composition of the placebo administered to the control group. Some authors omit its description altogether; others, in an endeavour to match the taste with that of glutamic acid, gave glycine (e.g. Kerr & Szurek, 1950; McCulloch, 1950) or included yeast extract (e.g. Milliken & Standen, 1951). It seems to have been taken for granted that these substances are indifferent, that the effects of glutamic acid are connected with its specific *in vitro* effects and that they are therefore just as specific. Nobody has so far troubled to test this point in cases of mental deficiency, but Rausch & Schwöbel (1949) described remarkable psychological effects when amino-acid mixtures were fed to patients suffering from chronic malnutrition. They found an improvement of calculating performance, memory and concentration; the patients showed signs of euphoria, their physical and mental inertia and apathy disappeared and their need of sleep decreased. Such results were not achieved with amounts of casein containing up to four times more nitrogen than the amino-acid mixture. What is particularly significant in this connection is the fact that the amino-acid mixture used contained no glutamic acid.

The effect of glutamic acid in hypoglycaemic coma has been shown to be equally unspecific, as it could be obtained with various other amino-acids.

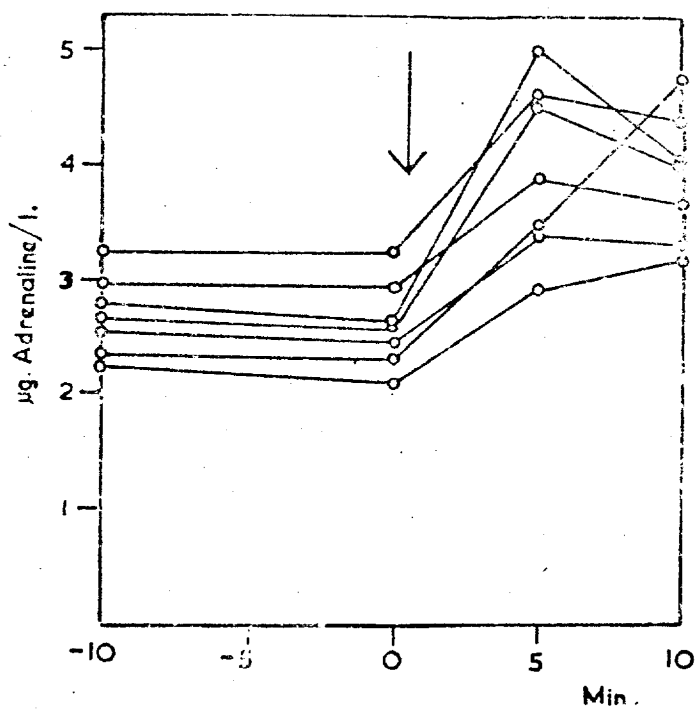

Fig. 2. Concentration of catechol amines (in terms of adrenaline) in the blood of patients during hypoglycaemic coma. At the time marked by the arrow 10 ml. 28·7% (w./v.) solution of monosodium glutamate was injected intravenously.

The effect of glutamic acid is not only unspecific, but is also unlikely to be due to a direct action on the brain cells, since the rate of transfer across the blood-brain barrier is very slow (Klein & Olsen, 1946; Schwerin, Bessman & Waelsch, 1950; Dawson, 1950). It has been suggested that the common factor in the clinical effects of glutamic acid is an adrenergic stimulation (Weil-Malherbe, 1950). The reciprocal correlation between the adrenergic system and the level of amino-acids in blood is well known and any sudden rise of the latter may be expected to produce a discharge of adrenergic amines. The effect of glutamic acid in hypoglycaemic coma may be reproduced by an intravenous injection of adrenaline, and the psychological and anticonvulsive effects by the administration of amphetamine. A new method for the estimation of catechol amines in blood has recently been developed (Weil-Malherbe & Bone, 1952) and with its aid the rise of adrenergic amines in blood following the injection of glutamic acid during hypoglycaemic coma, which has previously been demonstrated (Weil-Malherbe, 1949), could be confirmed on a quantitative basis. Fig. 2 shows the results obtained so far. The first two values represent the concentration of catechol amines in blood (in terms of adrenaline) before the injection of glutamic acid, the last two that after the injection. An increase of catechol amines was regularly observed (Weil-Malherbe, 1951).

In view of the ready availability of glutamic acid in the food and its high rate of synthesis in the tissues its effects cannot be explained as the repair of an absolute or relative deficiency. Moreover, as already mentioned, a direct effect of glutamic acid on the brain cells is improbable owing to the action of the blood-brain barrier. These difficulties resolve themselves if an adrenergic mechanism is assumed. The beneficial effects of glutamic acid therapy need not be denied merely because the premises on which it was founded turn out to be untenable. If one reads the detailed case reports either in the original papers of Zimmerman, Burgemeister & Putnam (1946, 1947) or, for instance, in the recent paper of Schwöbel (1950), one cannot escape the impression that in some cases glutamic acid treatment can cause dramatic personality changes. The individual response, however, seems to be very variable and the most responsive cases are probably comparatively rare. Any improvement of intelligence is presumably secondary to an improvement of the personality, resulting in a more favourable emotional attitude towards intellectual tasks. It is significant that a rise in the general level of activity and spontaneity, an increase of emotional stability and a higher degree of accessibility and co-operation have been reported by authors who found no statistically valid effects on test scores. The observations on the effect of glutamic acid treatment in catatonic schizophrenia indicate similar changes. Ewalt & Bruce (1948) report that those patients who were apathetic and easily fatigued to begin with showed an increase of motor activity and a greater interest in their environment and their personal appearance. Kitzinger, De Vere, Cartwright & Shapiro (1949) found an increase of emotional responsiveness, of productivity and motor control in those patients who initially showed a low level of activity, while those who started with a higher level of activity increased in hostility, excitability and tension.

GLUTAMIC ACID AND CEREBRAL CIRCULATION

The breathing of a CO_2-rich gas mixture tends to reduce petit mal attacks, but to bring on grand mal seizures (Lennox, Gibbs & Gibbs, 1936; Gibbs, Lennox & Gibbs, 1940). The effects of glutamic acid are very similar; while it inhibits the occurrence of petit mal attacks (Waelsch & Price, 1944) it may precipitate grand mal seizures (Zimmerman, 1949; Weil-Malherbe, unpublished observations). Carbon dioxide causes vasodilatation in the brain (Gibbs, Gibbs & Lennox, 1935; Kety & Schmidt, 1948) and the effects observed in epileptics are probably based on this action. By analogy a similar vasomotor effect of glutamic acid might be expected, although there is as yet no evidence to prove it. Such an effect would not be inconsistent with an adrenergic mechanism, since small doses of adrenaline are known to induce an increase of cerebral blood flow (Rein, 1937; Rothlin & Taeschler, 1951) and a dilatation of retinal vessels (Heuerkamp & Rittinghaus, 1950).

NUTRITIONAL FACTORS IN GLUTAMIC ACID METABOLISM

Reference has already been made to the neurological and psychiatric effects of pyridoxine deficiency and their possible connection with a decreased activity of the transamination and decarboxylation of glutamic acid in brain.

The epileptogenic effects of agene poisoning in dogs may be due to a disturbance of glutamine metabolism. The toxic agent has been identified as methionine sulphoximine, a compound structurally resembling glutamine (Bentley, McDermott & Whitehead, 1950). It inhibits the growth of *Leuconostoc mesenteroides* and the effect is reversed by glutamine (Heathcote & Pace, 1950). Brain slices from animals poisoned with methionine sulphoximine show a decreased capacity for the synthesis of acetylcholine and the *in vitro* addition of glutamine restores the synthesis to the normal level (Tower & Elliott, 1951). Methionine sulphoxide, a closely related compound, is an antagonist of glutamine in bacterial metabolism (Waelsch, Owades, Miller & Borek, 1946) and inhibits the synthesis of glutamine in brain extract (Elliott, 1951).

BLOOD LEVEL OF GLUTAMINE, GLUTAMATE AND KETOGLUTARATE IN PSYCHOTIC SUBJECTS

The values found for the concentration of glutamic acid and glutamine in normal blood have been reviewed by Waelsch (1951 *b*). Pond (1950) studied the distribution of plasma amino-acids on paper chromatograms, but found no abnormalities of pattern in various forms of mental disorder. It is, however, not possible to detect finer quantitative differences with this method. Munkvad (1950) using an enzymic method of analysis has reported a significant shift of the glutamine glutamic acid ratio in the plasma of psychotics, while the sum of the concentrations of the two substances remained practically unchanged. In acute schizophrenia and in acutely manic states the mean values of the ratio were found to be 10 times higher than the normal average. As the schizophrenic cases became more chronic the ratio tended to approach normal values and cases of more than 10 years' duration showed no deviation from the normal average. A common factor in acute mania and acute schizophrenia is a high degree of tension and excitement which may even underlie apparently stuporous states. It would be interesting if a connection could be established between the increase of the glutamine glutamic acid ratio and the increase in the activity of the autonomic nervous system.

Buscaino & Rapisarda (1948) claim to have shown an increase of ketoglutaric acid in the plasma of catatonic schizophrenics. The values published, however, show a large scatter and a statistical comparison with an adequate sample of control cases has not been carried out.

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